

The Fungal Vacuole: Composition, Function, and Biogenesis

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INTRODUCTION	266
VACUOLAR HYDROLASES	267
Synthesis and Processing.....	267
Transit through the secretory pathway: carboxypeptidase Y	267
Processing pathway	268
Proteinase A	269
Proteinase B.....	270
Aminopeptidase I	270
Trehalase	271
α -Mannosidase	271
Dipeptidyl aminopeptidase B	271
Alkaline phosphatase	272
ATPase	272
Sorting Signals	272
Carboxypeptidase Y.....	273
Proteinase A	273
Alkaline phosphatase	273
Dipeptidyl aminopeptidase B	274
Overproduction-induced mislocalization	274
Mutants Defective in Vacuolar Protein Sorting	274
<i>vpt</i> mutants	275
<i>vpl</i> mutants	276
<i>pep</i> mutants	278
Genetic complexity of the sorting pathway.....	278
VACUOLAR ATPase AND VACUOLE ACIDIFICATION	278
Vacuolar ATPase	278
Functions of Vacuole Acidification	279
Amino acid and ion antiport.....	279
Precursor maturation.....	280
Sorting.....	280
Assembly of ATPase	280
Mutations Affecting Vacuole Acidification.....	281
COMPARTMENTALIZATION OF METABOLITES	282
Role of the Vacuolar ATPase in Metabolite Transport	282
Amino Acid Transport and Storage.....	282
Inorganic Ion Transport and Storage	283
Polyphosphates	283
pH and Osmoregulation.....	284
Regulation of Transport	284
Vacuolar Storage Mutants.....	284
VACUOLE BIOGENESIS.....	285
CONCLUSION.....	286
ACKNOWLEDGMENTS.....	287
LITERATURE CITED.....	287

INTRODUCTION

The fungal vacuole is often described as an organelle that is analogous to the mammalian lysosome. Although this viewpoint is largely correct, it is also misleading, even though both the lysosome and the vacuole are acidic compartments which contain a variety of hydrolytic enzymes (1,

64, 87). Although macromolecular degradation is one obvious function of the fungal vacuole, it is also involved in a variety of additional cellular processes. The importance of the fungal vacuole in metabolite storage and in cytosolic ion and pH homeostasis, for example, is well documented (3, 31). In this regard, the fungal vacuole has greater similarity to the vacuole of plant cells (102). A number of reviews have focused on individual properties of the vacuole, including proteolysis (1, 64), metabolite transport (31), and acidification (3, 122). The purpose of this review is not only to discuss the current work on the fungal vacuole, but also to

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TABLE 1. Protein constituents of the vacuole

Name or abbreviation used in text	Protein designation	Gene designation
Soluble		
CPY	Carboxypeptidase Y, carboxypeptidase yscY	<i>PRC1</i>
PrA	Proteinase A, proteinase yscA	<i>PEP4, PRA1, PHO9</i>
PrB	Proteinase B, proteinase yscB	<i>PRB1</i>
API	Aminopeptidase I, aminopeptidase yscI, leucine aminopeptidase IV, aminopeptidase V, aminopolypeptidase, aminopeptidase III	<i>LAP4, APE1</i>
Trehalase	Trehalase	
Membrane associated		
α -Mannosidase	α -Mannosidase	<i>AMS1</i>
DPAP B	Dipeptidyl aminopeptidase B, dipeptidyl aminopeptidase yscV, X-prolyldipeptidyl aminopeptidase	<i>DAP2, DPP2</i>
ALP	Alkaline phosphatase	<i>PHO8</i>
ATPase	H ⁺ -translocating ATPase	<i>vma, VMA, VAT</i>

provide a more comprehensive picture of this complex organelle and reveal how it is integrally involved in a variety of cellular processes.

VACUOLAR HYDROLASES

Synthesis and Processing

The yeast vacuole has been viewed primarily as a degradative organelle because of the variety of hydrolase activities that have been localized to this compartment. Well-

characterized vacuolar hydrolases include proteinase A, proteinase B, carboxypeptidase Y, carboxypeptidase S, aminopeptidase I, aminopeptidase Co, dipeptidyl aminopeptidase B, repressible alkaline phosphatase, RNase, and α -mannosidase (see references 1, 64, and 153 for review). Abbreviations for these hydrolases, which will be used throughout this review, are noted in Table 1. The levels of many vacuolar hydrolases vary with the growth stage and nutrient supplies (50, 94, 111, 113, 149). These enzymes are derepressed under conditions of limiting glucose or nitrogen and tend to reach maximal levels as the cells approach the stationary phase. Vacuolar hydrolases have been implicated in several processes that can be viewed as long-term adaptations to changing nutritional conditions. These mostly involve general proteolysis, the degradation and reutilization of small peptides, and sporulation-associated protein degradation (1, 64, 163). Interestingly, there is little direct evidence that the vacuole is required for the turnover of aberrant and nonfunctional proteins (64, 163). Recent applications of molecular biological, genetic, and biochemical techniques to the study of vacuolar proteins have provided information on the biosynthesis, processing, and localization of many of these proteins.

Transit through the secretory pathway: carboxypeptidase Y. The most thoroughly studied vacuolar protein is carboxypeptidase Y (CPY). CPY has frequently been viewed as the typical model vacuolar protein. Although recent studies on a variety of hydrolases now suggest that there may be no such thing as a typical vacuolar protein, an analysis of CPY serves as a useful introduction to the biosynthesis (i.e., translation, transport, and proteolytic and/or glycosyl modifications) of many vacuolar proteins.

CPY is synthesized as an inactive precursor protein that, characteristic of proteins that transit through the secretory pathway (Fig. 1), translocates into the endoplasmic reticu-

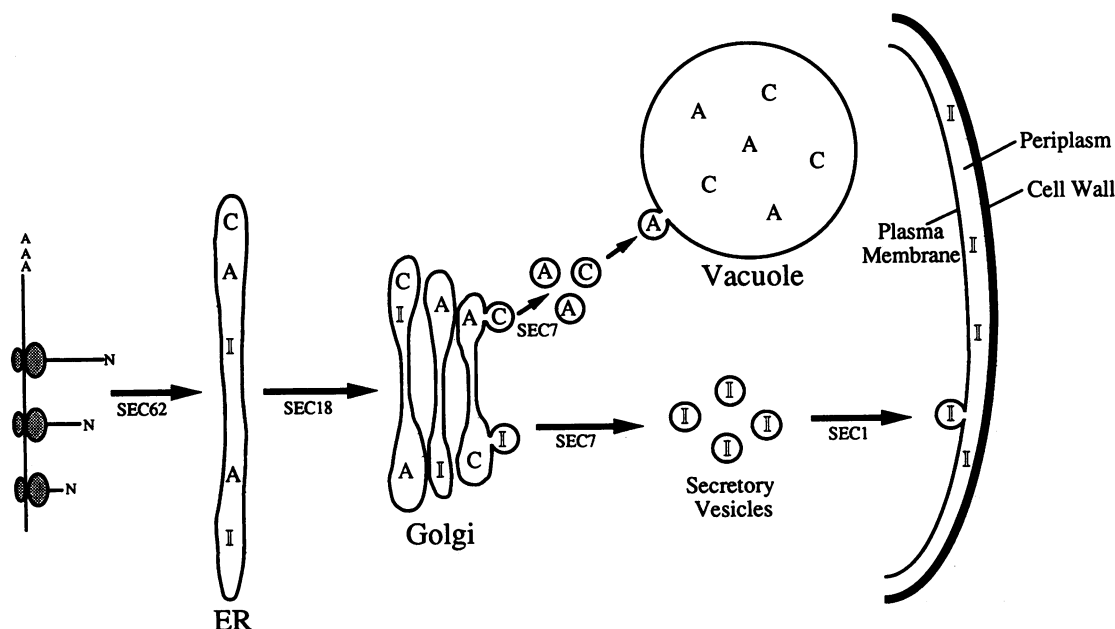


FIG. 1. The secretory enzyme invertase (I) and the vacuolar hydrolases CPY (C) and PrA (A) are synthesized in the cytoplasm and sequestered into the lumen of the ER, where they are modified with *n*-glycosidically linked core oligosaccharides. The proteins then transit to the Golgi complex, where further glycosyl modification takes place. Invertase is then packaged in secretory vesicles that deliver this enzyme to the cell surface, and CPY and PrA are targeted to the vacuole by a secretory vesicle-independent route. The *SEC* gene products are required for transport through the secretory pathway: *SEC62*, translocation into the ER (33, 34); *SEC18*, transit beyond the ER (123, 156); *SEC7*, transit beyond the Golgi complex (43, 123, 156); *SEC1*, secretion via secretory vesicles (123).

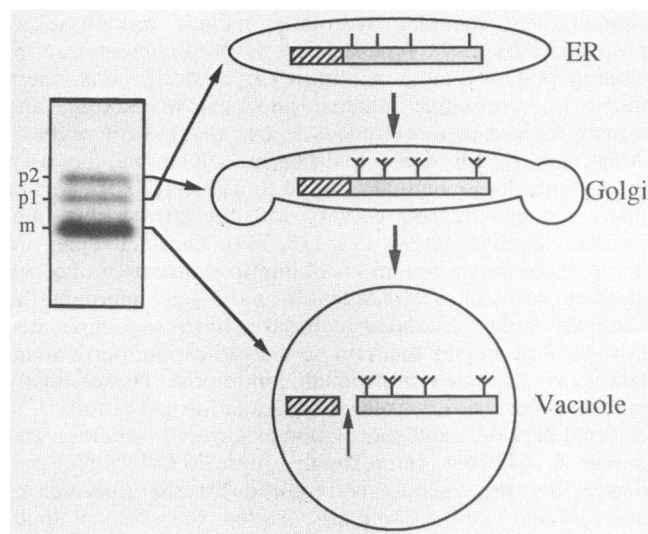


FIG. 2. Carbohydrate and proteolytic processing forms of CPY. As the precursor protein translocates into the ER, the signal peptide is proteolytically removed and the protein is core glycosylated (p1 form). During transit through the Golgi complex, additional carbohydrate modification takes place (p2 form). The protein is then targeted to the vacuole, where a propeptide segment is proteolytically removed, before or upon arrival, to generate the mature active enzyme (m form). Symbols: , propeptide; , mature enzyme. The approximate locations of the four N-linked oligosaccharide addition sites are indicated above the enzyme. Reprinted from UCLA Symp. Mol. Cell. Biol. (83a) with permission of the publisher.

lum (ER) as a result of the presence of an N-terminal signal sequence (11, 62). Temperature-sensitive mutations in the secretory pathway that block ER translocation, *sec61* and *sec62* (33, 34) (Fig. 1), cause the unglycosylated, signal sequence-containing form of CPY, termed preproCPY (54), to accumulate in the cytoplasm. In a wild-type strain, the 20-amino-acid signal peptide is proteolytically removed during or immediately after translocation (11, 62). Translocation into the ER is accompanied by an increase in molecular mass as the protein undergoes dolichol-mediated core glycosylation (54). The addition of core oligosaccharides, having the structure $(\text{GlcNAc})_2(\text{Man})_9(\text{Glc})_3$ (93), to the four sites in proCPY having the tripartite recognition sequence Asn-X-Thr, produces the 67-kilodalton (kDa) (54) form called p1 CPY (156) (Fig. 2). The addition of phosphate via phosphomonoester and -diester groups to the carbohydrate side chains also takes place in the ER (53, 152, 156, 166). The precise mechanism of transit from the ER to the Golgi complex is not known but is believed to occur via vesicular carriers. The dependence of transit on the *sec18* gene product (156) (Fig. 1) supports this prediction, since *sec18* has been shown to be homologous to the mammalian N-ethylmaleimide-sensitive fusion protein, a protein that appears to be involved in ER-to-Golgi transit and intra-Golgi vesicle fusion (10, 193). Passage of proteins through the yeast Golgi complex is not as well defined as it is in mammalian cells, because of the lack of good fractionation procedures for the yeast Golgi complex and the relatively simple carbohydrate modifications that take place in yeasts. A distinction can be made between early and late Golgi compartments by analyzing proteins that accumulate in the Golgi-blocked *sec7* mutant strain (43, 156) (Fig. 1). The proCPY that accumulates in a *sec7* mutant at the restrictive

temperature is modified with α -1,6-mannose-linked carbohydrates, but does not contain the α 1,3-mannose carbohydrate linkages that are present on the mature protein (43). In contrast to the long mannose outer chains characteristic of secreted yeast proteins, CPY and other vacuolar proteins contain oligosaccharide side chains that undergo limited elongation (see Sorting Signals). During transit through the Golgi complex, three of the four core oligosaccharides on CPY are elongated to produce carbohydrate side chains containing an average of 11 to 18 mannoses (166). The fully glycosylated Golgi precursor form, p2 CPY (156), has a molecular mass of 69 kDa (56). The sorting of proCPY from nonvacuolar proteins which also utilize the secretory pathway is believed to take place in the *trans*-Golgi (48). The final transport step, delivery to the vacuole, is again not well defined but is presumed to occur through the use of specific vesicular intermediates. These vesicles, however, are distinct from secretory vesicles involved in cell surface transport of secreted and plasma membrane proteins, since transit to the vacuole is not blocked in *sec1* mutant yeast cells which accumulate secretory vesicles at the nonpermissive temperature (156) (Fig. 1). Just before or upon arrival in the vacuole, the N-terminal propeptide segment of proCPY (56) is proteolytically removed, generating the active 61-kDa mature form (54). The half-time for the maturation process has been shown to be approximately 6 min (54).

Processing pathway. Many of the vacuolar hydrolases, including CPY, proteinase A (PrA), and proteinase B (PrB), are synthesized as inactive zymogens that contain propeptide segments (56) (Fig. 3). Maturation of proCPY, and other vacuolar proenzymes, is dependent on a functional *PEP4* (PrA) gene product (56). These precursor enzymes, including proCPY and proPrA, are likely to transit the same compartments in the early secretory pathway and utilize the same vesicle carriers for delivery to the vacuole, yet no processing of the precursors appears to occur until arrival of each of these proteins in the vacuole. The central role of PrA in the maturation of several vacuolar proenzymes led to the proposal of an activation mechanism that is triggered by the pH-dependent autoactivation of PrA (2, 195). This model is supported by the observation that processing of proCPY and proPrB in vitro has a pH optimum of 5.0 (103, 105). Recent determinations of the vacuolar pH and an analysis of processing in mutants with an altered vacuolar pH (see Vacuolar ATPase and Vacuole Acidification), however, do not presently support this model. The requirement of an additional vacuolar constituent for PrA-dependent processing, such as polyphosphate, may in part be the trigger that initiates vacuolar processing (105). Both genetic and biochemical evidence point to a role for PrA in the processing pathway (56, 105, 200). Strains with *pep4* mutations have extremely reduced levels of CPY activity and accumulate the p2 precursor form of the enzyme. In addition, purified PrA can activate proCPY in vitro in the absence of other known vacuolar hydrolases. Interestingly, the CPY that is processed by PrA alone migrates on sodium dodecyl sulfate-polyacrylamide gels with an apparent molecular mass slightly greater than that of the in vivo form (105). When this form is incubated in vitro in the presence of PrB, the authentic mature form of CPY is generated, suggesting a role for PrB in the processing pathway in vivo. This is further supported by the observation that the propeptide cleavage site between amino acids Asn-111 and Lys-112 is not a preferred substrate for PrA (105). The initial cleavage of proCPY by PrA is not required for the PrB-dependent processing in vitro (54, 105), and it is not clear whether both

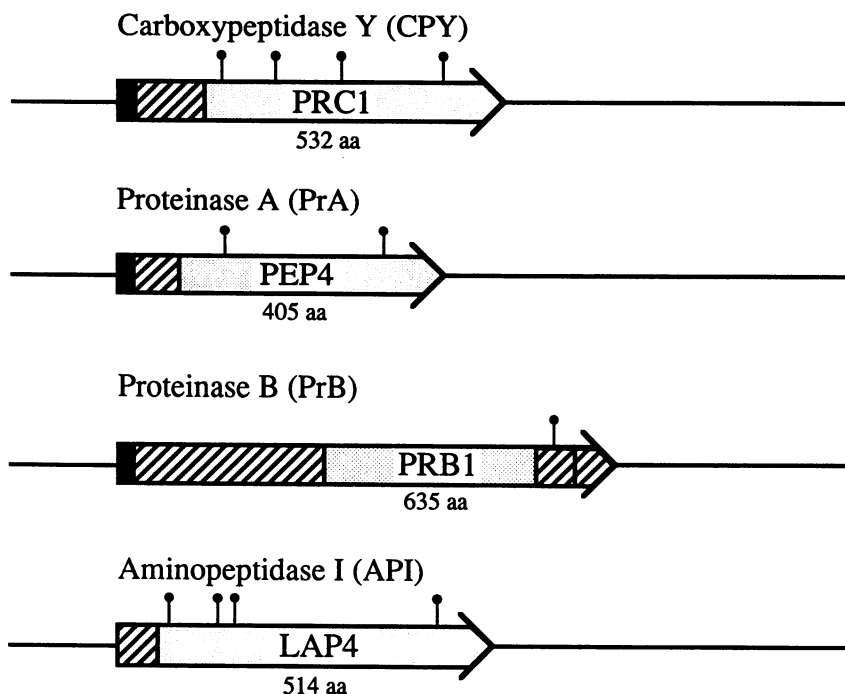
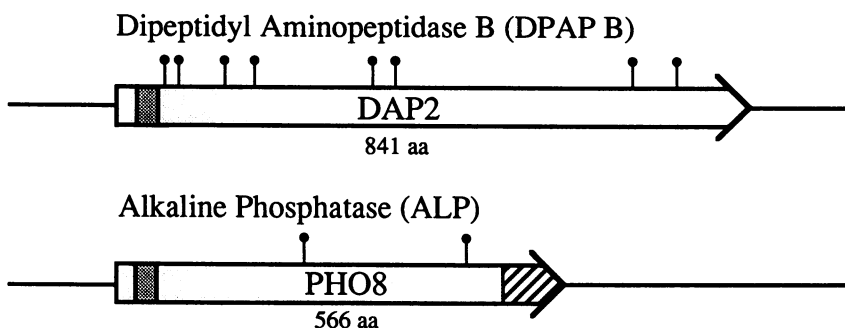
Soluble Vacuolar Hydrolases:Membrane-associated Vacuolar Hydrolases:

FIG. 3. Biosynthesis of vacuolar hydrolases. The genes are divided into the coding regions for the presumed signal peptide (■), the proteolytically cleaved propeptide sequences (▨), and the mature enzyme (□). Transmembrane domains within the mature enzyme are also shown (▤). The approximate positions of the asparagine-linked core oligosaccharides are indicated. The number of amino acids (aa) encoded by each gene is indicated below the gene designation. The schematic diagrams are based upon the nucleotide sequence information from the cloned *PRC1* (157, 171), *PEP4* (2, 195), *PRB1* (111, 114), *LAP4* (23, 30), *DAP2* (142) and *PHO8* (71, 72) genes.

normally occur in vivo. Mature PrB, when overproduced, is in fact capable of processing vacuolar proCPY in the absence of PrA (65). Conversely, CPY is still activated in PrB-defective strains, indicating that PrA can activate proCPY directly in vivo (56). Finally, the facts that PrB can activate other vacuolar hydrolases and its specific activity in crude extracts shows a gene dosage dependence on the *PEP4* gene may indicate a complexity within the processing pathway that allows for considerable regulation (66).

Proteinase A. PrA is similar to CPY in that it is a soluble vacuolar glycoprotein hydrolase that is initially synthesized as a larger inactive precursor (Fig. 3). The extreme N terminus of preproPrA contains a sequence that fits with the consensus for signal sequences and is predicted from the rules of von Heijne (174) to be cleaved by signal peptidase

between amino acids 22 and 23. In agreement with this prediction, the first 23 amino acids of preproPrA are able to functionally replace the signal sequence of the normally secreted enzyme invertase (80). Full-length, unglycosylated preproPrA accumulates in *sec62* mutant yeast cells which are defective in translocation across the ER membrane. The signal peptide is cleaved at the level of the ER, and the cleaved form of the protein accumulates in *sec18* (ER-to-Golgi transit defective) yeast cells at the restrictive temperature. N-linked core oligosaccharides are added in the ER at two sites (106) corresponding to preproPrA residues Asn-144 and Asn-345 (2, 195). As with CPY, the core glycosylated species is referred to as p1 PrA. During transit through the ER and Golgi complex, the core oligosaccharides are trimmed and extended, resulting in an increased molecular

mass of 48 to 52 kDa. The size increase of this p2 form is consistent with its undergoing the same type of limited carbohydrate modification seen with CPY. During or upon delivery to the vacuole, the propeptide is cleaved between amino acids Glu-76 and Gly-77 of preproPrA (2, 145) to generate the 42-kDa mature active enzyme (Fig. 3). The half time for the maturation process, approximately 6 min, is identical to that seen for CPY, suggesting that both precursors utilize the same set of transit intermediates en route to the vacuole. As stated above, proPrA may mature autocatalytically. PrA activation is not dependent on CPY, PrB, or carboxypeptidases (105) but the participation of another, as yet unidentified protein, cannot be ruled out.

Proteinase B. The analyses of the biosynthesis of CPY and PrA provided a deceptively simple and cohesive model for the modification, processing, and transport of soluble vacuolar hydrolases. Initial studies of PrB added to this common model; a precursor form of PrB of approximately 40 kDa was identified that underwent a *PEP4*-dependent maturation step to generate a mature enzyme of approximately 30 kDa (104, 106). This maturation occurred with the same kinetics as seen for proCPY (112), suggesting that PrB traversed the secretory pathway in much the same way as CPY and PrA did. One difference was that whereas the PrB precursor showed a tunicamycin-sensitive molecular mass shift, the mature protein did not (106). Since it was known that mature PrB was a glycoprotein (85), this suggested the presence of hydroxyl-linked (O-linked) carbohydrate groups. A major insight into the biosynthesis of PrB was afforded by the cloning of the PrB structural gene, *PRB1* (111). Sequencing of the *PRB1* gene revealed an open reading frame of 635 amino acids encoding a protein of approximately 70 kDa, substantially larger than the previously identified precursor (114). The first 19 residues of the deduced amino acid sequence have the characteristics of a hydrophobic signal sequence, supporting the prediction that PrB travels through the secretory pathway. In addition, the N-terminal 20 amino acids of precursor PrB can functionally replace the invertase signal sequence, allowing translocation of invertase into the ER and subsequent secretion from the cell (D. Klionsky and S. Emr, unpublished observations). Surprisingly, a comparison of the amino acid sequence from purified mature PrB with that of the deduced amino acid sequence from the *PRB1* gene indicated that the mature protein begins at amino acid 281 (starting from the initiation codon) (114). The segment between the end of the signal peptide and the start of the mature protein, an additional 261 amino acids, must therefore correspond to a propeptide domain. The large size of this propeptide and the fact that it is highly charged make it unclear whether the propeptide translocates across the ER membrane. The predicted molecular mass shift resulting from the proteolytic removal of this N-terminal propeptide would not yield a protein the size of mature PrB, but, rather, one the size of the 40-kDa precursor that accumulates in *pep4* cells. This led to the conclusion that a second processing event must occur during the maturation of PrB that involves the removal of a C-terminal polypeptide segment. Since this part of the protein contains a site for N-linked glycosylation, removal of this segment by PrA cleavage would explain the difference in tunicamycin sensitivity seen between the precursor and mature forms of PrB. A detailed analysis of the different forms of PrB present in conditional secretion-deficient mutants confirmed the predictions of a more complicated processing pathway than that seen for CPY or PrA (103, 112) (Fig. 3). In vitro (103) and in vivo experiments involving a rapid pulse-chase analysis (112)

revealed a PrB precursor of >70 kDa. This precursor could be accumulated in *sec61* and *sec62* mutant yeast cells and was unaffected by tunicamycin, indicating that it represented preproPrB which had not translocated into the ER (103, 112). Shortly after translocation of all or part of preproPrB into the ER, the N-terminal 280-amino-acid propeptide is removed to generate an intermediate form of approximately 39 kDa (41.5 kDa in reference 103). Since this cleavage still takes place in *sec18* cells at the nonpermissive temperature, it is presumed to occur in the ER (103, 112). This initial cleavage (it is not known whether the signal peptide is removed independently) also occurs in a *pep4* strain, which demonstrates that it is not PrA dependent. During transit of the peptide through the Golgi complex, elongation of the N-linked (but probably not the O-linked) oligosaccharide side chain results in a small increase in molecular mass (103, 112). A detailed analysis of strains containing mutations that affect glycosyl modification, *alg6*, *gls1*, and *mnn1*, confirmed that proPrB has only one N-linked chain (112). The Golgi form of PrB identified in these experiments is identical to the previously identified (106) 40-kDa precursor found in *pep4* mutant cells (42 kDa in reference 103). Although mature vacuolar PrB has a molecular mass of 31 kDa (33 kDa in reference 103), it appears that this form of the enzyme results from two successive cleavage reactions. The penultimate proteolytic step is carried out by PrA and results in the conversion of the 40-kDa Golgi intermediate to a 37-kDa form (112). An unidentified protease then converts the 37-kDa species to the final 31-kDa mature PrB. Interestingly, there is evidence that PrB itself is involved in the final activation step(s), since inhibitors of PrB block this reaction (103). This processing scheme involves the action of at least one and possibly two uncharacterized proteases for the eventual maturation of a vacuolar protease. The presence of O-linked sugars and the removal of a C-terminal polypeptide also reveal that the processing pathway of PrB is quite different from that of CPY and PrA. Since the large N-terminal propeptide is proteolytically removed early in the secretory pathway, its role is unclear. It is possible that it remains associated with PrB after cleavage and plays some role in sorting or in inhibition of PrB activity (103, 112).

PrB from the yeast *Candida albicans* has been purified and shown to be very similar to that from *Saccharomyces cerevisiae* on the basis of physical and enzymatic properties (41). This enzyme appears to be located in the vacuole; a specific protein inhibitor resides in the cytoplasm, as is seen with CPY, PrA, and PrB from *S. cerevisiae* (42, 94).

Aminopeptidase I. Yeast cells contain a variety of aminopeptidases, but only one of these, aminopeptidase I (API), is known to be localized in the vacuole (44). API is a soluble metalloexopeptidase that is strongly activated toward leucine substrates by Zn^{2+} (108). The native enzyme is an approximately 569- to 640-kDa multimeric glycoprotein composed of identical subunits of 50 to 53 kDa (23, 108, 167). API activity is *PEP4* dependent (167) and is derepressed by growth under conditions of limiting glucose or nitrogen (44), typical of other soluble vacuolar hydrolases. The structural gene coding for API, *LAP4*, was recently cloned and sequenced (23, 30). The open reading frame encodes a 514-amino-acid protein. Analysis of the N-terminal amino acid sequence of the mature protein indicates the presence of a 45-residue prosequence (23) (Fig. 3). API is synthesized as a 57-kDa precursor (23) that contains four potential sites for N-linked glycosylation (23, 30). The calculated molecular mass of the mature protein lacking carbohydrate is 44.8 kDa (108), making it unlikely that all four sites are glycosylated or

that they have the standard oligosaccharide structure found on vacuolar proteins. Since two of the potential glycosylation sites have the less frequently used Asn-X-Ser sequence (23, 30, 114), it seems likely that these sites are not glycosylated *in vivo*. Resolution of this question will require more definitive experiments. Interestingly, the extreme N terminus of the API precursor lacks a sequence that fits with the consensus hydrophobic signal sequence. A 16-residue stretch of the prosequence, however, contains both hydrophobic and hydrophilic residues that can be arranged in an amphiphilic α -helix (23, 30). The function of this unusual prosequence and the means by which API translocates into the ER have yet to be ascertained.

Trehalase. Trehalase is the only enzyme known to be involved in the catabolism of trehalose, a neutral disaccharide that is used as a storage carbohydrate in a variety of fungi (reviewed in reference 164). *S. cerevisiae* possesses two distinct trehalase activities, a cyclic AMP-dependent cytosolic enzyme and a vacuolar enzyme (75, 98, 190). Since the cytosolic trehalase is active only when phosphorylated, compartmentalization plays an important role in trehalase metabolism; trehalase is located primarily in the cytosol (75), whereas the constitutively active trehalase is sequestered within the vacuole (190). The vacuolar enzyme is a glycoprotein with a pH optimum of 4 to 5 and has an apparent molecular mass of 215 kDa based on gel filtration (99). Vacuolar trehalase appears to transit through the early stages of the secretory pathway. Delivery to the vacuole is blocked in *sec18* and *sec7* mutant yeast cells at the nonpermissive temperature but, like in other vacuolar hydrolases, is unaffected in a *sec5* (accumulates secretory vesicles) mutant (52). The activity of vacuolar trehalase is also *PEP4* dependent, suggesting that it is synthesized as a precursor form that is processed in a PrA-dependent manner (51). The vacuolar trehalase is probably not essential, since a *pep4* strain is able to grow as well as a wild-type strain on trehalose (51).

α -Mannosidase. At present the biosynthesis of α -mannosidase is not fully understood, even though it is the classic marker enzyme of the vacuole membrane. α -Mannosidase is localized in a particulate fraction and displays the same type of carbon catabolite repression and sporulation-induced increase in activity seen with other vacuolar enzymes (132). Recently, α -mannosidase was purified from yeast vacuoles (197). Although the enzyme activity appears to be membrane associated, its solubilization characteristics suggest that it may be a peripheral membrane protein or an ecto-type integral membrane protein with a small hydrophobic region that is attached to the inner surface of the vacuole membrane (197). This may also be true for the vacuolar α -mannosidase from *Neurospora crassa*, since only 22% of the activity remains associated with purified vacuolar membranes (172). The molecular mass of the native enzyme from *S. cerevisiae* was determined to be 560 kDa, which represents active isoforms composed of three polypeptides of 107, 73, and 31 kDa. The 107- and 73-kDa polypeptides are closely related, as determined by peptide mapping and cross-reactivity with specific antisera. The appearance of the 73-kDa species relative to the 107-kDa polypeptide increases with time in the stationary phase of growth, suggesting that the former is produced as the result of a specific proteolytic conversion (197). The same is presumably true of the 31-kDa polypeptide, since it is present in approximately equimolar amounts with the 73-kDa polypeptide. The structural gene for yeast α -mannosidase, *AMS1*, has been cloned and sequenced (89, 196) and was shown to encode the 107-kDa polypeptide

(196). A strain carrying a chromosomal disruption of *AMS1* does not synthesize either the 107- or 73-kDa polypeptides, confirming that the smaller polypeptide is proteolytically derived from the 107-kDa primary gene product (196). An analysis of the deduced amino acid sequence does not reveal the presence of an N-terminal signal sequence or any transmembrane domains that may function as internal signal sequences (196). The lack of a large hydrophobic region fits with the observation that α -mannosidase activity is extractable by Na_2CO_3 at high pH (197). The apparent absence of a signal sequence raises an intriguing question about the biosynthesis of α -mannosidase. Since the enzyme activity is present within the vacuolar lumen, it must translocate across some membrane during its biosynthesis. α -Mannosidase, however, is not mannosylated even though it has seven potential sites for N-linked glycosylation, suggesting that it may not transit through the secretory pathway (196). This would explain the insensitivity of α -mannosidase to the sorting defects exhibited by most of the vacuolar protein-sorting mutants (see Mutants Defective in Vacuolar Protein Sorting). α -Mannosidase may be delivered to the vacuole and translocated across the vacuolar membrane directly from the cytoplasm (196). This would be similar to the direct translocation of proteins targeted for degradation into the lumen of lysosomes in mammalian cells (24).

Dipeptidyl aminopeptidase B. Yeast express at least two dipeptidyl aminopeptidases (DPAPs) that are associated with a particulate fraction (158, 159). One of these activities represents the product of the *STE13* gene, dipeptidyl aminopeptidase yscIV (DPAP A), which is involved in α -factor pheromone processing (67, 159) and is presumably localized to a late Golgi compartment. The other enzyme, dipeptidyl aminopeptidase yscV (DPAP B), is encoded by the *DAP2* gene (159) and is associated with the vacuolar membrane (12). DPAP A and DPAP B activities are easily distinguished because of the thermolability of the latter. DPAP B is the first vacuolar membrane protein for which detailed biosynthetic data were determined. In contrast to the soluble vacuolar hydrolases, DPAP B activity is not *PEP4* dependent or enhanced by incubation of a crude extract at pH 5.0 (158), suggesting that it does not contain a propeptide domain (Fig. 3). This lack of *PEP4* dependence is similar to that seen with α -mannosidase. The nucleotide sequence of the *DAP2* gene was recently determined (142) and found to encode an 841-amino-acid polypeptide of predicted molecular mass 96,429 Da. Although DPAP B and α -mannosidase activities cofractionate (12), DPAP B is not removed from the vacuole membrane by treatment with Na_2CO_3 at pH 11.5 (142), indicating that it is an integral membrane protein. A pulse-chase analysis with antiserum specific to DPAP B reveals that the protein is initially made as 110- and 113-kDa species in the ER (142). The difference in size between the two ER forms is due to heterogeneous core glycosylation, since both species migrate with a molecular mass of 96 kDa after treatment with endoglycosidase F. Titration with endoglycosidase F indicates that at least five of the eight potential N-linked glycosylation sites are used. This type of heterogeneous glycosylation is similar to that seen with core-glycosylated invertase (40, 156). The ER forms of DPAP B chase into a 120-kDa form in the Golgi complex (142). This molecular mass shift suggests that the oligosaccharide chains on DPAP B undergo the same type of limited extension seen with other vacuolar hydrolases. As expected from earlier studies (158), DPAP B does not undergo a detectable *PEP4*-dependent cleavage and the enzyme is in fact active in the ER (142). Localization by immunofluorescence confirms that

the protein is associated with the vacuolar membrane in wild-type yeast cells (142). In contrast, *sec18* and *sec7* mutant strains accumulate nonvacuolar forms of DPAP B as revealed by both immunofluorescence and sodium dodecyl sulfate-polyacrylamide gel analysis. Like the soluble vacuolar hydrolases, DPAP B is transported through the early stages of the secretory pathway. A hydropathy analysis of the deduced amino acid sequence of DPAP B showed the presence of a single hydrophobic domain near the amino terminus (142). This is predicted to result in the orientation of DPAP B as a type II integral membrane protein, with the C terminus inside the lumen and an N-terminal cytoplasmic domain of approximately 29 amino acids.

Alkaline phosphatase. The nonspecific alkaline phosphatase (ALP) is the best-characterized yeast alkaline phosphatase. It is a repressible enzyme regulated in response to phosphate levels through a system of genes including *PHO4*, *PHO80*, *PHO81*, and *PHO85* (see reference 133 for a review). Its activity is also modulated posttranslationally by the *PEP4* gene product. ALP has a molecular mass of 130 kDa and is composed of two identical subunits of molecular mass 66 kDa (131). It is a glycoprotein containing N-linked oligosaccharides which constitute 8% of its total weight (131), suggesting the presence of two glycosidic side chains. ALP is encoded by the *PHO8* gene (73, 165) which has been cloned (72) and sequenced (71). The deduced amino acid sequence predicts a protein of 566 amino acids, with two potential sites for N-linked glycosylation at Asn-268 and Asn-401 (71). ALP activity has been localized to the vacuole (9, 26, 110, 191), but there have been differing reports concerning its association with the vacuolar membrane. Cytochemical studies have localized ALP to the inner side of the vacuolar membrane (9, 26), and a particulate alkaline phosphatase activity was found to cofractionate with vacuolar membranes (110). This enzyme, however, was characterized as being different from the previously identified soluble ALP (131). Subsequent studies suggest that the two enzymes are identical (26). Recently, a detailed biochemical characterization of ALP has been carried out (82). ALP is initially made as a larger precursor that is matured in a PrA-dependent manner with a half-time of approximately 6 min. The ALP precursor transits through the same part of the secretory pathway as many other vacuolar proteins and can be accumulated in *sec18* and *sec7* mutant yeast cells at the restrictive temperature. Both the precursor and mature forms of ALP are membrane associated. Analysis of ALP solubility in the presence of saponin and Na_2CO_3 indicate that it transits through the secretory pathway and resides in the vacuole as an integral membrane protein. A hydropathy analysis of the deduced amino acid sequence of ALP reveals the presence of a single hydrophobic domain near the N terminus, preceded by a stretch of basic amino acids (71, 82). Since ALP lacks a standard signal sequence at its extreme N terminus, this hydrophobic domain presumably functions as both an ER translocation signal and a membrane anchor. This would predict an orientation of ALP as a type II integral membrane protein (Fig. 3). A type II orientation has been confirmed by protease protection experiments, which indicate the presence of an N-terminal cytoplasmic tail on ALP (82). ALP is unusual in that it is the only characterized vacuolar membrane protein that undergoes a PrA-dependent cleavage. The ALP propeptide was shown to be removed from the C terminus of the precursor protein. This is consistent with earlier observations that a mutated ALP with a C-terminal truncation expressed activity independently of the *PEP4* gene (71, 72).

ATPase. A vacuolar membrane ATPase has been identified and partially purified in yeasts and *Neurospora crassa*. Because of the substantial amount of data available on this enzyme, and its importance in vacuole function, it is discussed separately below (see Vacuolar ATPase and Vacuole Acidification).

Sorting Signals

All of the vacuolar proteins that have been characterized with regard to their transport properties, with the possible exception of α -mannosidase (see above), travel to the vacuole via the secretory pathway (80, 82, 103, 112, 142, 156) (Fig. 1). They transit from the ER to the Golgi complex, undergoing both proteolytic and glycosyl modifications, and can be accumulated in these organelles along with normally secreted proteins in *sec* mutants that, at the nonpermissive temperature, are transport defective. The *sec* mutants that block the movement of secretory proteins after the Golgi complex do not affect the delivery of vacuolar proteins to the vacuole (142, 156). This suggests that secretory and vacuolar proteins travel together through the ER and Golgi complex before being sorted from one another for final delivery to their distinct subcellular destinations. Since all of the proteins that use the secretory pathway also use the same set of transit organelle intermediates, there must be signals within the proteins themselves that allow them to be sorted and targeted in a precise and efficient manner. One of the major modifications made to proteins upon translocation into the ER is the addition of core oligosaccharides, which are further modified in the Golgi complex, resulting in a structure for vacuolar proteins of the form $(\text{GlcNAc})_2(\text{Man})_{11-18}$. These oligosaccharide side chains are often also modified with phosphodiester groups (53). Secretory proteins such as invertase receive the same type of core oligosaccharides in the ER, but undergo a more extensive elongation of these side chains in the Golgi complex, where 50 to 100 mannose residues may be added to each side chain (6, 162). One explanation for this differential modification would be that secretory and vacuolar proteins travel separately through the secretory pathway and are accessible to different mannosyl transferases in the Golgi complex. The specific type of glycosyl modification might mark each protein for delivery to the vacuole or the cell surface. This would be analogous to the mechanism used for the sorting of some mammalian lysosomal proteins that are modified with mannose 6-phosphate, allowing recognition and lysosomal delivery through interactions with the mannose 6-phosphate receptor (reviewed in references 87 and 173). Four lines of evidence, however, argue against a direct role for glycosyl modifications in vacuolar protein sorting. First, it has been demonstrated that CPY, PrA, and ALP can be delivered to the vacuole and matured in the presence of tunicamycin, a drug that blocks the addition of N-linked oligosaccharides (26, 80, 152, 156). Second, hybrid proteins consisting of segments of vacuolar proteins fused to invertase are efficiently delivered to the vacuole, even though they undergo the same type of extensive carbohydrate elongation as is seen with wild-type invertase (62, 80). Third, certain hybrid proteins that lack any oligosaccharide addition sites on the vacuolar part of the hybrid are delivered to the vacuole (62, 80, 83). Fourth, overproduction of CPY (157) leads to secretion of this protein without the addition of long mannose outer chains (see Overproduction-Induced Mislocalization). These observations suggest that (i) vacuolar proteins are accessible to the same compartments of the secretory pathway as are

secreted proteins, (ii) the type of glycosylation received does not specify the ultimate subcellular destination, and (iii) the elimination of glycosylation, and hence phosphorylation, does not prevent proper sorting. This is supported by recent data showing the correct vacuolar localization of CPY which has been altered to remove one or more of the N-linked glycosylation sites by site-specific mutagenesis (J. R. Winther, T. H. Stevens, and M. C. Kielland-Brandt, personal communication). This apparent lack of a role for glycosylation in the sorting process suggests that any targeting information expressed by the vacuolar proteins must be contained within the polypeptide chains themselves.

Carboxypeptidase Y. Two independent approaches were undertaken simultaneously to characterize the sorting information in CPY. The first of these relies on the use of gene fusions to the *SUC2* gene. The *SUC2* gene codes for invertase, a glycoprotein enzyme that is easily assayed and is competent for delivery through the secretory pathway. Invertase normally resides in the periplasm, where it catalyzes the hydrolysis of extracellular sucrose. Secretion of invertase is believed to occur by a default pathway; there is no evidence that active signals beyond the N-terminal signal peptide are required for its localization in the periplasm (62, 76, 171, 186). These properties make invertase a useful marker enzyme with which to monitor vacuolar protein sorting. Plasmid vectors have been constructed which contain a truncated *SUC2* gene missing the N-terminal signal sequence and the first two amino acids of the mature protein (62, 83). Portions of the gene of interest, in this case *PRC1*, were cloned in front of and in frame with the *SUC2* gene, so that hybrid proteins are produced that retain invertase activity. When the N-terminal 20 amino acids of preproCPY are fused to invertase, the hybrid protein is secreted from the cell (62). This indicates that the first 20 amino acids of preproCPY can functionally replace the invertase signal sequence. This hybrid construct is competent to translocate into the ER and transit through the secretory pathway. In contrast, larger hybrid proteins containing 50 to 433 amino acids of preproCPY are efficiently retained within the cell, and subcellular fractionation studies demonstrate that they cofractionate with isolated vacuoles (62). This shows that a vacuolar targeting signal in preproCPY resides within the N-terminal 50 amino acids.

The N-terminal 50 amino acids of preproCPY are sufficient to direct invertase to the vacuole. To determine whether this region is also required for vacuolar delivery of the wild-type protein and to precisely define the location of the sorting information, a second approach was taken that used mutations in the wild-type *PRC1* gene (62). This approach was also used to independently map a vacuolar sorting signal in CPY (171). A deletion of amino acids 21 to 50 (62) or smaller deletions in the vicinity of amino acids 25 to 31 (171) of preproCPY result in missorting and secretion of an otherwise wild-type precursor protein. In addition, random chemical and site-directed mutagenesis of the *PRC1* gene identified a single amino acid change, Gln-24-to-Lys, that was sufficient to cause the same missorting phenotype (171). Subsequent mutational analyses of a region spanning the vacuolar sorting information, residues 18 to 34, confirmed the importance of Gln-24 and identified three additional residues that may contribute to the sorting signal (147). The mutant CPY that was secreted from yeast cells was present as the p2 form, indicating that it transits through the ER and Golgi compartments of the secretory pathway similar to the wild-type CPY protein. This fact, along with the observation that secretion is blocked in *sec1* (accumulates secretory

vesicles) mutant yeast cells under restrictive conditions, supports the hypothesis that sorting occurs at a late stage in the Golgi complex (171). The proCPY that is secreted can be matured and activated, indicating that missorting is not due to gross structural changes or misfolding (171). These mutational analyses suggest that amino acids 24 to 31 of preproCPY are critical for efficient sorting. Taken together with the gene fusion studies, these results indicate that the N terminus of proCPY is both sufficient and necessary for vacuolar delivery.

Proteinase A. A similar analysis was carried out with PrA to determine the location and important features of its vacuolar-sorting determinant (80). Hybrid proteins containing 76 amino acids from the N terminus of preproPrA fused to invertase were efficiently delivered to the vacuole. In contrast, a hybrid protein with 61 N-terminal amino acids of preproPrA was inefficiently delivered to the vacuole, with the majority of the protein being secreted from the cell. The N-terminal 76 amino acids of PrA define the signal sequence and propeptide (2, 145), indicating that vacuolar-sorting information in PrA is located within the propeptide domain, similar to CPY, and suggesting that this may be a common theme for soluble vacuolar hydrolases. The N-terminal prosequence of API (23), which is presumably cleaved in a PrA-dependent reaction (167), may also contain sorting information. Deletions within the PrA propeptide cause dramatic instability of the mutated protein, suggesting that an additional role of the propeptide is to allow the precursor protein to fold into its normally protease-resistant form (80). This instability, however, has made it difficult to assess the effects of these mutations on PrA sorting or to further define the PrA sorting signal. A comparison of the amino acid sequences of proPrA and proCPY does not reveal any strong sorting signal consensus sequence that is shared by these two proteins. The most likely explanations for this are (i) the two proteins contain distinct vacuolar-targeting information and use different sorting components even though they share a common delivery pathway and (ii) the vacuolar-sorting signal may contain secondary and/or tertiary structural information. Recent data indicate that glycosylation of PrA may be required for efficient vacuolar sorting (J. R. Winther and M. C. Kielland-Brandt, personal communication). The implications of this observation are not clear, however, since PrA-invertase hybrid proteins lacking the oligosaccharide addition sites on PrA are efficiently delivered to the vacuole (80).

Alkaline phosphatase. ALP is the first vacuolar membrane protein for which detailed sorting information has been determined (83). Gene fusions between *PHO8* and *SUC2* indicate that the N-terminal 52 amino acids of ALP are sufficient to direct the vacuolar delivery of invertase. This segment contains just the cytoplasmic tail and most of the transmembrane domain of ALP. A shorter hybrid protein lacking the N-terminal hydrophobic domain remains in the cytoplasm. This confirms that one role of the transmembrane domain is to act as an internal uncleaved signal sequence, allowing translocation into the ER. Replacement of the first 53 amino acids from wild-type ALP with a functional signal sequence results in missorting and secretion of approximately 50% of the protein (83), confirming the requirement of the N-terminal domain for vacuolar sorting. The remainder of the protein is not delivered to the vacuole but instead accumulates in the ER, possibly because some of the ALP folds improperly and aggregates. The propeptide of ALP maps to the C terminus of the precursor protein (see Synthesis and Processing; Alkaline Phosphatase) (Fig. 3). ALP

is therefore different from CPY and PrA in that its sorting information is not contained in a luminal propeptide segment that is removed from the protein after it reaches its final destination, the vacuole.

An analysis of ALP sorting has also provided some insight into potential differences between targeting information in soluble and membrane-associated vacuolar proteins. Although both types of proteins transit through the same stages of the secretory pathway and are delivered to the vacuole with similar kinetics (see Synthesis and Processing), ALP exhibits some differences in the characteristics of the sorting process. The most notable of these is an apparent pH independence for vacuolar delivery. Precursor ALP is matured with normal kinetics in the presence of bafilomycin A₁ (83), a drug that inhibits the vacuolar ATPase and causes missorting of soluble vacuolar hydrolases (see Vacuolar ATPase and Vacuole Acidification). This is most easily explained by the location of the ALP sorting signal in the cytoplasmic and/or transmembrane domains of the protein; changes in the luminal pH of the vacuole or Golgi complex are less likely to affect interactions with these sequences. The importance of this observation is that it points out the likely interaction of ALP with a different sorting component(s), such as a receptor, than is used by the soluble hydrolases. This reliance on unique sorting components is also suggested by the relative insensitivity of ALP to the missorting defects exhibited by certain of the vacuolar protein-sorting mutants (see Mutants Defective in Vacuolar Protein Sorting; Vacuolar Protein-Targeting (*vpt*) Mutants).

Dipeptidyl aminopeptidase B. Preliminary evidence indicates that a short N-terminal region of DPAP B is sufficient to direct invertase to the vacuole (C. Roberts and T. Stevens, unpublished observation). This would indicate that, similar to ALP, the vacuolar sorting information in DPAP B is contained within the cytoplasmic tail and/or transmembrane region (Fig. 3).

Overproduction-induced mislocalization. Some insight into the mechanism of vacuolar protein sorting is afforded by the observation that overproduction of CPY-invertase hybrid proteins or, more importantly, wild-type CPY, leads to missorting and secretion of these proteins (7, 157). The missorted CPY is secreted as the p2 form, and secretion is blocked in *secl* mutant yeast cells at the nonpermissive temperature, indicating that secreted proCPY must transit through the late secretory pathway (157). These results could be explained by the saturation of a limiting component that is required either for recognizing and sorting CPY directly or for modifying CPY such that it can be subsequently sorted and delivered. This type of result is indicative of a saturable receptor-mediated sorting process; production of CPY above normal physiological levels leads to secretion of much of the excess precursor. Since glycosylation of the secreted precursor is normal (157), the machinery responsible for glycosyl modifications is able to handle the higher levels of substrate. This further shows that glycosylation does not determine the subcellular location of vacuolar proteins. A similar result is seen with overproduction of PrA-invertase hybrid proteins (Klionsky and Emr, unpublished observation) and wild-type PrA (145). Interestingly, overexpression of PrA does not significantly affect the sorting of CPY (145) and, similarly, overproduction of CPY does not cause secretion of PrA (157). This may indicate the use of different receptors, consistent with the lack of homology in the vacuolar sorting signals. Alternatively, overproduction-induced secretion may result from some other effect of overexpression, such as the production of sorting-defi-

cient aggregates. At present, no definitive genetic or biochemical evidence exists for the presence of a receptor for vacuolar proteins. It is interesting that overexpression of *AMS1*, the gene encoding α -mannosidase, does not lead to the presence of enzymatic activity at the cell surface (89).

Mutants Defective in Vacuolar Protein Sorting

The highly compartmentalized nature of the eucaryotic cell suggests that mechanisms exist to effectively target and deliver cellular proteins from their site of synthesis in the cytoplasm to their appropriate destination. Analyses of the delivery of several vacuolar proteins, including CPY, PrA, and ALP, have indicated that specific structural determinants present within the proteins themselves are responsible for their observed vacuolar localization (see Sorting Signals). The loss of this sorting information, through mutational alteration, results in the missorting of the mutated vacuolar protein to the cell surface (62, 80, 83, 171). These observations indicate that the delivery of proteins to the yeast vacuole is an active process. Since vacuolar and other secretory-pathway proteins transit through the same compartments, specific components within the cell must function to distinguish vacuolar proteins from the rest of the secretory traffic and to ultimately deliver these proteins to the vacuole. As discussed above (see Synthesis and Processing; CPY), vacuolar proteins enter into the secretory pathway at the ER and follow an intracellular path similar to that of lysosomal proteins in mammalian cells (86, 87). The genetic data are consistent with the conclusion that the vacuolar protein sorting reaction occurs at a late stage within the Golgi complex and suggest that vacuolar proteins transit from the ER to the Golgi together with proteins destined for secretion or assembly into the plasma membrane. At some point within the Golgi, or shortly after exit from this organelle, the vacuolar proteins are sorted away from the rest of the secretory traffic and are targeted to the vacuole.

Recent genetic studies of vacuolar protein localization in *S. cerevisiae* have led to the isolation of a large number of yeast mutants that exhibit defects in vacuolar protein sorting (7, 143, 144, 146). Studies of these mutants, and the genes affected in them, have indicated that the delivery of proteins to the vacuole is a rather complex process, which requires the coordinated participation of a large number of cellular functions. Two independent genetic approaches have been used in efforts to obtain yeast mutants defective for vacuolar protein targeting (7, 143, 144, 146). Both selections are based upon the assumption that defects in the vacuolar protein-sorting machinery will result in the mislocalization of vacuolar proteins to the cell surface. This was a logical extension of earlier genetic studies which had demonstrated that alteration of the *cis*-acting sorting signals within CPY resulted in its secretion from yeast cells (62, 171). In addition, the overproduction of CPY in wild-type yeast cells results in the appearance of precursor CPY at the cell surface (157). These data suggest that a failure to properly sort vacuolar proteins would result in their secretion from the yeast cells. Both of these genetic approaches have been successful, resulting in the isolation of a large number of mutants which secrete vacuolar proteins. The *vpt* mutants (for vacuolar protein targeting defective) were isolated by a gene fusion approach which took advantage of the efficient vacuolar localization of a CPY-invertase hybrid protein (7, 143). In the second scheme, yeast *vpl* mutants (for vacuolar protein localization defective) were identified by directly selecting for the presence of CPY enzymatic activity at the cell surface (144, 146).

TABLE 2. Genetic overlap between the *vps*, *pep*, and other related sets of mutants

<i>vps</i> mutant	<i>vpt</i> mutant	<i>vpl</i> mutant	<i>pep</i> mutant	Others
<i>vps1</i>	<i>vpt26</i>	<i>vpl1</i>		
<i>vps2</i>		<i>vpl2</i>		
<i>vps3</i>	<i>vpt17</i>	<i>vpl3</i>	<i>pep6</i>	
<i>vps4</i>	<i>vpt10</i>	<i>vpl4</i>		
<i>vps5</i>	<i>vpt5</i>	<i>vpl5</i>	<i>pep10</i>	
<i>vps6</i>	<i>vpt13</i>	<i>vpl6</i>	<i>pep12</i>	
<i>vps7</i>		<i>vpl7</i>	<i>pep15</i>	
<i>vps8</i>	<i>vpt8</i>	<i>vpl8</i>		
<i>vps9</i>	<i>vpt9</i>			
<i>vps10</i>	<i>vpt1</i>			
<i>vps11</i>	<i>vpt11</i>	<i>vpl9</i>	<i>pep5</i>	<i>end1, vam1</i>
<i>vps12</i>	<i>vpt12</i>			
<i>vps13</i>	<i>vpt2</i>			
<i>vps14</i>	<i>vpt14</i>			
<i>vps15</i>	<i>vpt15</i>			
<i>vps16</i>	<i>vpt16</i>			<i>vam9</i>
<i>vps17</i>	<i>vpt3</i>		<i>pep21</i>	
<i>vps18</i>	<i>vpt18</i>		<i>pep3^a</i>	<i>vam8</i>
<i>vps19</i>	<i>vpt19</i>			
<i>vps20</i>	<i>vpt20</i>	<i>vpl10</i>		
<i>vps21</i>	<i>vpt21</i>			
<i>vps22</i>	<i>vpt22</i>	<i>vpl14</i>		
<i>vps23</i>	<i>vpt23</i>	<i>vpl15</i>		
<i>vps24</i>	<i>vpt24</i>			
<i>vps25</i>	<i>vpt25</i>	<i>vpl12</i>		
<i>vps26</i>	<i>vpt4</i>		<i>pep8</i>	
<i>vps27</i>	<i>vpt27</i>			
<i>vps28</i>	<i>vpt28</i>	<i>vpl13</i>		
<i>vps29</i>	<i>vpt6</i>			
<i>vps30</i>	<i>vpt30</i>			
<i>vps31</i>	<i>vpt31</i>			
<i>vps32</i>	<i>vpt32</i>			
<i>vps33</i>	<i>vpt33</i>		<i>pep14^a</i>	<i>slp1, vam5, cls14</i>
<i>vps34</i>	<i>vpt29</i>			
<i>vps35</i>	<i>vpt7</i>			
<i>vps36</i>		<i>vpl11</i>		
<i>vps37</i>		<i>vpl16</i>		
<i>vps38</i>		<i>vpl17</i>		
<i>vps39</i>		<i>vpl18</i>		
<i>vps40</i>		<i>vpl19</i>		

^a Recent data have indicated that, in contrast to a previous report (144), the *pep* mutants marked with an asterisk exhibit the indicated overlaps (J. Robinson, S. Emr, R. Preston, J. Zhang and E. Jones, unpublished observations). No overlap has been detected between *pep1*, *pep2*, *pep4*, *pep7*, *pep9*, *pep11*, *pep13*, *pep16*, and the 40 *vps* complementation groups (143, 144). The relationship between the remaining *vam* mutants and the *vps* and *pep* sets of mutants has not yet been determined.

Not surprisingly, the *vpt* and *vpl* mutants obtained from these schemes exhibit very similar mutant phenotypes. Considerable genetic overlap exists between these two sets of mutants and, in all, the *vpt* and *vpl* mutants define 40 unique complementation groups (143, 144). The *vpt* and *vpl* complementation groups have recently been consolidated and are now collectively referred to as *vps*, for vacuolar protein sorting defective (Table 2). A third set of mutants, *pep*, originally identified as defective for CPY enzymatic activity (63), have recently been shown to exhibit defects in the localization of several soluble vacuolar hydrolases, including CPY (144). Complementation analysis with the *vps* and *pep* sets of mutants has again demonstrated extensive genetic overlap. Presently, all of these mutations affecting vacuolar protein sorting have been placed into 47 unique complementation groups (Table 2). The following sections discuss the identification and characterization of these three sets of mutants.

***vpt* mutants.** Early gene fusion studies demonstrated that specific N-terminal sequences of CPY were sufficient to direct the normally secreted enzyme, invertase, to the yeast vacuole (7, 62). These fusions retained invertase activity, but this normally periplasmic activity was now intracellular, sequestered within the vacuolar compartment (7, 62). Emr and colleagues have taken advantage of these observations to develop a genetic selection for yeast mutants defective in the localization of these fusion proteins (7, 143). Briefly, yeast Δ *suc2* strains, which lack secreted invertase activity, are unable to grow on media containing sucrose as the sole fermentable carbon source. Δ *suc2* yeast strains which harbor a single-copy plasmid encoding a vacuolar CPY-invertase fusion protein continue to exhibit a sucrose-negative (*Suc*⁻) growth phenotype, because yeast cells are unable to transport sucrose across the plasma membrane and into the vacuole, where the invertase activity is now sequestered (7, 62). Such *Suc*⁻ yeast strains were placed on media containing sucrose as the sole fermentable carbon source, and mutants which could grow on sucrose (*Suc*⁺) were selected (7, 143). Subsequent analyses of these *Suc*⁺ mutants demonstrated that all of the mutations perturb the localization of the CPY-invertase hybrid protein and result in secretion of invertase activity. More importantly, the intracellular sorting and delivery of several wild-type vacuolar proteins are also defective in each of these *Suc*⁺ mutants (see below). More than 600 *vpt* mutants were isolated and characterized. The recessive *vpt* mutations have been assigned to at least 33 *vpt* complementation groups (143).

Invertase assays with the *vpt* mutants demonstrate that all of the mutations result in the secretion, to various extents, of the gene fusion-encoded invertase activity (7, 143). This extracellular activity is not due to cell lysis as there is no increase in the periplasmic levels of two cytoplasmic proteins, α -glucosidase and glyceraldehyde-3-phosphate dehydrogenase, in the *vpt* mutants. In addition, many of the *vpt* mutants secrete more than 80% of this CPY-invertase fusion protein, a level much too high to be consistent with cell lysis, as these *vpt* cells exhibited near wild-type growth rates (7, 143). Since the *vpt* mutants display normal levels and rates of protein secretion, their defects appear to be specific for vacuolar sorting. If the CPY-invertase fusion protein was using normal host functions during its transit to the vacuolar compartment, *vpt* mutations might also be expected to affect the localization of wild-type CPY. In all *vpt* mutants examined, CPY accumulates as a Golgi-modified (p2) precursor form. The majority of this p2 CPY was secreted by mutant cells into the extracellular media fraction. The extent of the CPY mislocalization closely mirrored the CPY-invertase fusion defect (143). In at least one case, this appearance of CPY at the cell surface was shown to be dependent upon *SEC1* gene function (7). This result, together with the apparently normal ER and Golgi modification of the mislocalized CPY, suggests that the normal secretion pathway is being used during *vpt*-dependent secretion of CPY.

The *vpt* sorting defects extend beyond CPY to other soluble vacuolar hydrolases. In most *vpt* mutants, the processing and localization of both PrA and PrB are abnormal (143). In general, the accumulation of proPrA and proPrB can be correlated with the processing defects seen with CPY. However, the extent of secretion of the precursor forms of PrA and PrB is not as great as that observed for proCPY in the same mutants (143). The site of accumulation of the cell-associated PrA and PrB precursor molecules is not known. The *vpt* mutations therefore appear to be pleio-

tropic, affecting the delivery of several different soluble vacuolar hydrolases.

Another important question concerning the *vpt* mutants was whether they affected the localization of vacuolar membrane proteins in addition to soluble proteins. In mammalian cells, lysosomal membrane proteins are not modified with mannose 6-phosphate and do not utilize the mannose 6-phosphate receptor, indicating that at least some components of the sorting machinery are different (reviewed in reference 87). The targeting of yeast vacuolar membrane proteins was initially examined by assaying for α -mannosidase activity in *vpt* mutant cells (7, 143). α -Mannosidase is a classic marker enzyme for the yeast vacuolar membrane (132). The α -mannosidase active domain is thought to reside within the vacuolar lumen, and therefore mislocalization to the cell surface should result in the appearance of detectable cell surface activity. The majority of *vpt* mutants do not show a significant increase in external α -mannosidase activity, suggesting that the localization of this membrane protein is not significantly perturbed in these mutants (7, 143). Recent studies suggesting that α -mannosidase may be directed to the vacuole via a mechanism that is independent of the secretory pathway (see Synthesis and Processing) could provide an explanation for the relative insensitivity of this protein to the *vpt* defects. However, mutants with mutations in four different *vpt* complementation groups (*vpt11*, *vpt16*, *vpt18*, and *vpt33*) do exhibit significant α -mannosidase activity at the cell surface (143) (see below). Recently, the sorting of another vacuolar membrane protein, ALP, has been analyzed in the *vpt* mutants (82). ALP is an integral constituent of the vacuolar membrane and appears to transit through the same early stages of the secretory pathway as CPY (see Synthesis and Processing). In this study, the processing of proALP to a mature species was used as an indicator of vacuolar delivery. In most *vpt* mutants analyzed, there was some accumulation of proALP, although the extent of the ALP processing defect was relatively minor when compared with the processing and localization defects observed with CPY (82). In addition, since *vpt* mutants mis-sort PrA, an enzyme involved in ALP maturation, defects resulting in reduced processing of ALP may not result from missorting of this membrane protein. *vpt11*, *vpt16*, *vpt18*, and *vpt33* cells, however, exhibited a complete block in ALP maturation; this was the same subset of *vpt* mutants which was found to have cell surface α -mannosidase activity (143). This subset of *vpt* mutants constitutes a distinct class, in which the mutant cells appear to lack a normal vacuolar compartment (8) (see below). This analysis suggests that ALP is less sensitive to the sorting defects of most *vpt* mutations than are the soluble vacuolar hydrolases. This might indicate the presence within yeast cells of sorting components which are specific for vacuolar membrane protein localization. However, it should be pointed out that ALP maturation, and not specifically sorting, was assessed in this study. It must still be demonstrated that the processed ALP is indeed in the vacuole in these *vpt* mutants.

Many of the *vpt* mutants exhibit extreme defects in the sorting and/or processing of multiple vacuolar hydrolases. Such severe sorting defects might be expected to compromise other vacuolar functions or to affect the biogenesis of this organelle. To assess the structural integrity of the vacuolar compartment in the *vpt* mutants, an extensive morphological analysis, using both light and electron microscopy techniques, was carried out (8). The morphology of the vacuole in yeast cells can be easily visualized by using fluorescent dyes which accumulate specifically within this

compartment (100, 136, 179). Both fluorescein isothiocyanate and an endogenous fluorophore which accumulates within the vacuoles of *ade2* yeast cells were used in this study. Wild-type yeast cells, when visualized with either of these vacuole-specific dyes, are observed to possess one to three relatively large vacuoles per cell (8, 179). The majority of *vpt* mutants, representing 26 complementation groups, exhibit this wild-type staining pattern and have been designated class A mutants (8). Class B mutants, representing three other complementation groups, exhibited a fragmented vacuole morphology. Multiple small vacuolelike structures are present within each cell. Identical results were obtained when each mutant was analyzed by electron microscopy (Fig. 4). The vacuoles present within class B mutants might represent intermediates in vacuole biogenesis or, alternatively, may be by-products of the fragmentation of a larger vacuolar structure. Members of the final four *vpt* complementation groups, class C mutants, exhibited the most severe morphological defects. No significant intracellular staining was observed with either fluorophore, suggesting that class C cells may lack a vacuolar compartment (8). Instead, class C mutants accumulated small vesicles and a variety of abnormal membranous organelles within their cytoplasm (8) (Fig. 4). The precise origin or function of these structures is not presently known. It is possible that some of these structures represent remnants of a vacuolar compartment. The lack of a normal vacuole in these cells may suggest that the gene products defined by the class C mutants are involved in the regulation of the biogenesis of this organelle. In this case, the severe sorting defects might be due to the lack of an appropriate target structure. However, the lack of a vacuolar compartment might, instead, be a consequence of the extreme sorting defects associated with class C mutations. To choose the correct alternative, we require a better understanding of the primary lesion in the class C mutants. It is interesting that class C mutants, which lack a normal vacuolar compartment, are viable but exhibit a temperature-sensitive growth phenotype even though the severe morphological and sorting defects are seen at both temperatures (143). This might suggest that most vacuolar functions are nonessential for growth, at least at the lower temperatures. Alternatively, class C cells might retain residual levels of vacuolar activities, possibly in the accumulated membrane vesicles, which are sufficient for vegetative growth.

The CPY-invertase fusion selection scheme has been successful in identifying many gene functions which are required, directly or indirectly, for the proper sorting of proteins to the yeast vacuole (7, 143). Two other genetic approaches (see below) have also identified components of the cell necessary for this sorting process.

***vpl* mutants.** When CPY is overproduced in wild-type yeast cells, precursor CPY is detected at the cell surface (157). A fraction of this extracellular proCPY is processed to an active form by an unknown protease in a *PEP4*-independent reaction (157). This observation has formed the basis for a genetic selection used by Rothman et al. to obtain yeast mutants defective in the vacuolar localization of CPY (144, 146). This genetic selection also takes advantage of the observation that CPY possesses the major yeast proteolytic activity capable of cleaving the dipeptide *N*-carbobenzoxyl-L-phenylalanine-L-leucine (CBZ-PheLeu) to liberate free leucine (88, 194). Leucine auxotrophs are able to grow on medium which contains CBZ-PheLeu as the sole source of leucine as long as CPY activity is present (64, 146). *pep4* yeast cells, which possess no CPY activity (66), are unable

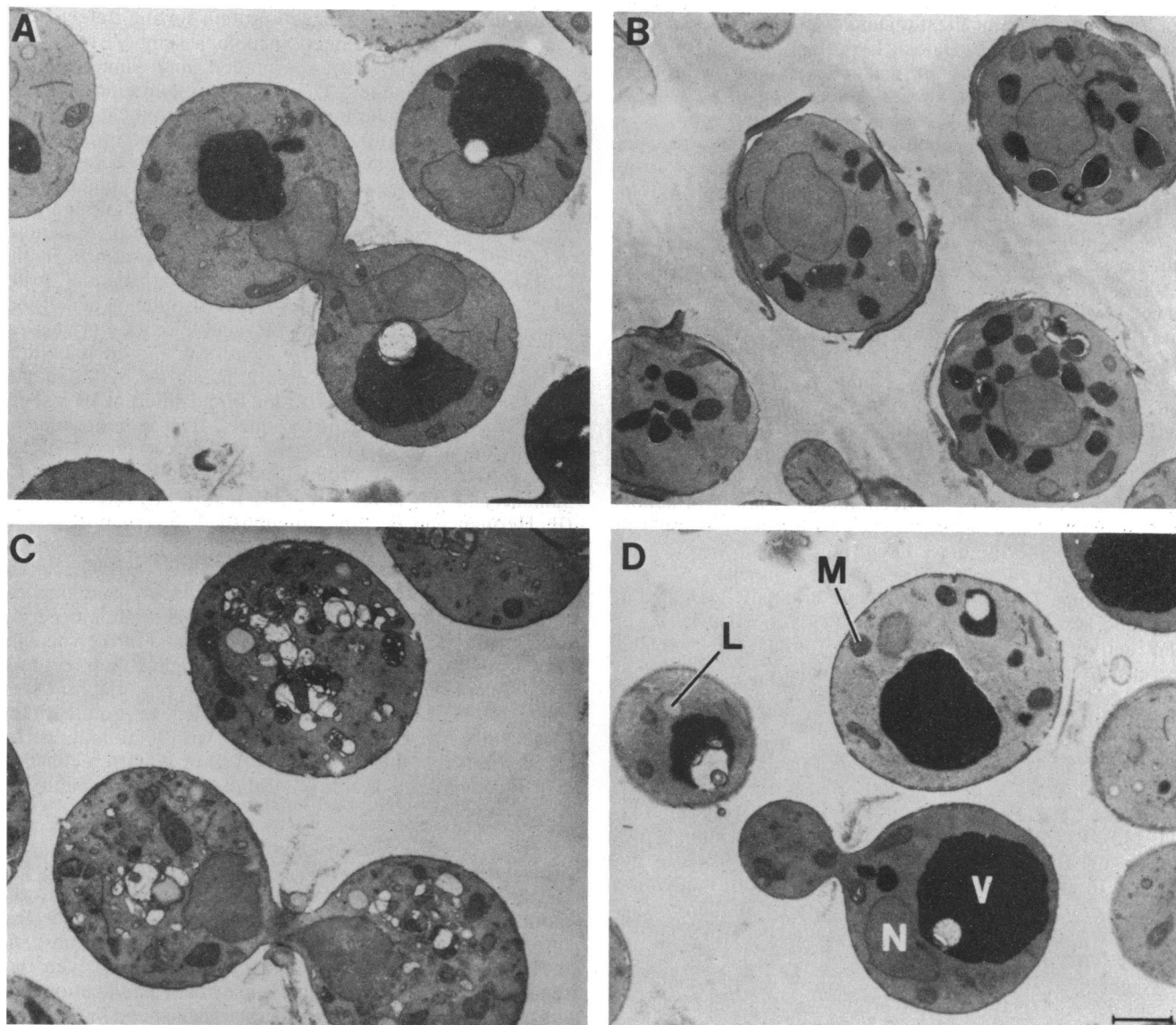


FIG. 4. Morphology of *vpl* mutant strains. *vpl* mutant strains with class A (A), class B (B) or class C (C) morphology and the isogenic wild-type strain SEY6210 (D) were prepared for electron microscopy (21) and processed by the reduced osmium-thiocarbohydrazide-reduced osmium procedure (192). Cells were then washed, dehydrated with ethanol, and embedded in LR white. Thin sections were stained with lead citrate (140). Abbreviations: V, vacuole; M, mitochondria; N, nucleus; L, lipid droplet. Bar, 1 μ m.

to utilize the CBZ-PheLeu dipeptide molecule, and, hence, *pep4 leu2* cells are unable to grow on the above medium (146). Therefore, by plating *pep4 leu2* yeast cells (expressing normal levels of CPY) onto medium containing CBZ-PheLeu as the sole leucine source, it was possible to select for yeast mutants which aberrantly localize CPY to the cell surface. A large number of *vpl* mutants, both spontaneously arising (144) and appearing after ethyl methanesulfonate-induced mutagenesis (146), were isolated in two separate studies. It is interesting that very different allele distributions were obtained in the two studies (144, 146). The recessive *vpl* mutations define at least 19 *vpl* complementation groups.

All of the *vpl* mutants, by definition, possess extracellular CPY activity (146). Therefore, it was not surprising that CPY was detected in an extracellular fraction of all *vpl* mutants examined (144, 146). Many of the *vpl* mutants secrete as much as 80 to 90% of their total CPY, and this was shown

not to result from cell lysis or CPY overproduction. The fact that *vpl*-mediated secretion of CPY is dependent upon *SEC1* gene function further supports this notion (146). This dependence on *SEC1* gene function also suggests that the route followed by proCPY to the cell surface is the same as that used by normally secreted proteins, such as invertase. In fact, normal protein secretion appears to be unaffected in the *vpl* mutants. Invertase glycosylation and delivery to the cell surface occur with near-wild-type kinetics in *vpl* mutants (146).

The *vpl* mutations also affect the delivery and/or maturation of other soluble vacuolar proteins (146). A significant proportion of PrA (up to 60%) is secreted from *vpl* cells, but, as is also observed with the *vpl* mutants (143), some of the proPrA remains cell associated (146). In addition, PrB enzymatic activity is reduced in *vpl* mutants, suggesting that its delivery is also defective in these cells. Therefore, *vpl*

mutations affect the localization and/or processing of at least three different luminal vacuolar hydrolases, CPY, PrA, and PrB. The localization of one vacuolar membrane protein, α -mannosidase, did not appear to be affected in the *vpl* mutants (146).

The intracellular morphology of the *vpl* mutants was analyzed by both Nomarski interference optics and electron microscopy (144, 146). Mutants with mutations in four different *vpl* complementation groups, *vpl1*, *vpl5*, *vpl9*, and *vpl19*, exhibit aberrant vacuolar morphologies; the mutant cells possess multiple, small vacuolelike organelles in place of the normal, large vacuolar compartments usually observed in wild-type cells (144). These structures were stained with the fluorescent dye quinacrine, indicating that they are acidic organelles and are most probably related to the vacuole. Therefore, these *vpl* mutants exhibit morphological defects similar to those seen with class B *vpt* mutants (8). In fact, complementation analyses indicate that significant overlaps exist between these two groups of mutants (143, 144) (see below). In addition to fragmented vacuoles, *vpl1*, *vpl9*, and *vpl19* mutant cells accumulate a variety of abnormal membrane-enclosed organelles within their cytoplasm (144, 146). Unlike the class C *vpt* mutants, however, each still possesses a vacuole or vacuolelike organelle.

pep mutants. The *pep* mutants were originally identified in a genetic screen for yeast mutants with reduced levels of CPY activity (63). Many of the *pep* mutants also exhibit decreased levels of PrA and PrB enzymatic activities. Seventeen *pep* complementation groups have been reported (63, 64), and one gene, *PEP4*, has been cloned and shown to encode PrA (2, 195). The *vpl* and *vpt* mutants display a phenotype that is very similar to that of the *pep* mutants; the *vas* mutant cells exhibit greatly reduced levels of cell-associated CPY, PrA, and PrB enzymatic activities (7, 143, 144, 146). In these mutants, the decreased enzymatic activities result from the mislocalization of the protease zymogens to the cell surface, where proteolytic activation occurs very inefficiently. This phenotypic similarity suggested that some of the *pep* mutants might be deficient in vacuolar enzyme activities because of the mislocalization of these enzymes to the cell surface. All of the *pep* mutants, except *pep4*, display vacuolar protein-sorting defects (144). A significant fraction of the total CPY and PrA protein is detected in an extracellular fraction of these *pep* mutants. Therefore, the *VPT*, *VPL*, and *PEP* gene products all appear to define a similar set of intracellular protein-sorting functions.

Genetic complexity of the sorting pathway. The delivery of proteins to the vacuole presumably involves a number of distinct reactions, which must be precisely regulated both spatially and temporally. Specific cellular components must recognize the vacuolar proteins, sort them away from the rest of the secretory protein traffic, and package them into specific transport vesicles that ultimately must recognize and fuse with the vacuole. If the sorting components, such as a putative receptor protein, are to be reused for multiple sorting cycles, additional cellular functions would be required for the recycling process. The genetic complexity would again increase if vacuolar proteins are delivered via a prevacuolar compartment such as an endosome, which might also receive endocytic traffic from the cell surface. Therefore, the list of potential activities and structures required for transport between the Golgi complex and the vacuole can easily accommodate the large number of gene products presently implicated by the genetic studies as having a role in vacuolar protein sorting and delivery.

In addition to the vacuolar protein-sorting defects, *vps* mutations appear to affect vacuole assembly, organellar acidification, cell growth, sporulation, and osmoregulation (8, 143, 144, 146, 148). Therefore, *vps* mutations affect a wide variety of cellular functions. At least in the instances of vacuole biogenesis and acidification, it seems important to ask whether these phenotypes are a secondary consequence of the sorting defects or whether they correspond to the primary defects in *vps* cells. The extreme vacuole morphological defects seen in several *vps* mutants, for example, might suggest a role for these *VPS* gene products in the biogenesis or maintenance of the wild-type vacuolar structure. Therefore, *vps* mutations might also define gene functions necessary for a variety of vacuole-related processes. An understanding of this sorting pathway and the individual *VPS* gene functions will be greatly facilitated by the molecular isolation and characterization of the different *VPS* genes and their respective gene products. The cloning and sequencing of one of the *VPS* genes, *VPS15*, has revealed that the predicted protein product exhibits significant sequence similarity to the serine-threonine family of protein kinases (P. Herman and S. Emr, unpublished observations). This might suggest that protein phosphorylation-dephosphorylation reactions are responsible for controlling specific steps in this sorting pathway. The cloning, sequencing, and localization of other *VPS* and *PEP* gene products should permit additional insights into their possible role(s) in the vacuolar protein-sorting reaction. An in vitro system which reconstitutes Golgi-to-vacuole transit will also be essential for determining the biochemical function of each gene product in the sorting process. Recent observations with semi-intact perforated yeast cells suggest that this type of in vitro reconstitution assay may be possible (T. Vida and S. Emr, unpublished observations).

VACUOLAR ATPase AND VACUOLE ACIDIFICATION

The fungal vacuole is considered to be analogous to the mammalian lysosome mainly because of two similar features: they both contain a variety of hydrolytic enzymes, and they are acidic organelles. It is only recently, however, that the functions associated with compartment acidification, and the components responsible for establishing and maintaining the vacuolar pH, have been elucidated.

Vacuolar ATPase

A vacuolar ATPase has been identified and partially purified from *S. cerevisiae* (68, 69, 168, 191), *Saccharomyces carlsbergensis* (96, 128), and *N. crassa* (16, 18). The vacuolar ATPases from these different organisms have certain common features and are differentiated from the mitochondrial F_0F_1 and plasma membrane ATPases on the basis of pH optima, subunit composition and structure, and sensitivity to inhibitors (reviewed in references 14, 18, 69, and 122). (i) Vacuolar ATPases have pH optima of approximately 7.0 to 7.5 (18, 68, 128). (ii) The enzyme complex has a molecular mass of approximately 400 to 500 kDa (16, 57, 169) and consists of at least three different types of subunits: two major polypeptides of approximately 70 and 60 kDa and a *N,N'*-dicyclohexylcarbodiimide (DCCD)-binding protein of 15 to 20 kDa (16, 96, 168), all of which are highly conserved. Minor polypeptides that may also be structural subunits have been observed in all three organisms (16, 69, 96). (iii) The vacuolar ATPases are insensitive to oligomycin, azide, and vanadate and are inhibited by *N*-ethylmaleimide, KNO_3 , KSCN, and bafilomycin A_1 (17–19, 68, 95, 96, 168).

Recent sequencing data and improved purification of the vacuolar ATPase have led to several revisions in subunit designations. The designations that are used in this review, along with previous or alternative designations, indicated in parentheses are as follows: *S. cerevisiae* 69 kDa (70) (subunit A [119], subunit *a*, 89 kDa, 67 kDa [57, 168], *S. carlsbergensis* 75 kDa [96]), *N. crassa* 67 kDa (20) (70 kDa [20]), *S. cerevisiae* 57 kDa (119) (subunit B [119], subunit *b*, 64 kDa [168], 60 kDa [70, 147], *S. carlsbergensis* 62 kDa [96]), *N. crassa* 57 kDa (13) (62 kDa, 60 kDa [13, 16]), *S. cerevisiae* 16 kDa (120) (subunit *c*, 19.5 kDa [168], 20 kDa [57], 17 kDa [70], *S. carlsbergensis* 9 kDa [96]), *N. crassa* 16 kDa (16, 20). The functions of the different ATPase subunits are not well characterized. The 69-kDa *S. cerevisiae* and 67-kDa *N. crassa* polypeptides are proposed to contain the catalytic site for ATP hydrolysis. These subunits bind radioactive ATP analogs such as 8-azido-ATP and are labeled by inhibitors of ATPase activity such as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole and *N*-ethylmaleimide (16, 169). The gene encoding this subunit from *N. crassa*, *vma-1*, and *S. cerevisiae*, *VMA1*, has been cloned, and sequence analysis reveals that it is particularly homologous to the β subunit of F_0F_1 ATPases, which is known to contain the enzyme active site (3, 20). The *S. cerevisiae* *VMA1* gene contains an internal coding region for a nonhomologous peptide insert that is presumably removed by an unidentified splicing mechanism (3). The 57-kDa polypeptide is inferred to be a regulatory nucleotide-binding protein by analogy to the homologous beet tonoplast 57-kDa polypeptide (69, 101). The gene encoding this subunit has been cloned from both *N. crassa* (designated *vma-2* [13]) and *S. cerevisiae* (designated *VAT2* [119, 147] and *VMA2* [3]). This subunit shows homology to the α subunit of F_0F_1 ATPases, also a regulatory nucleotide-binding protein, and the plasma membrane ATPase of the archaeobacterium *Sulfolobus acidocaldarius*. These relationships have evolutionary implications for the origin of the vacuolar ATPase (46, 120, 122). The 16-kDa polypeptide is likely to be involved in forming the proton channel. This polypeptide binds DCCD (16, 69, 96, 168), resulting in a block in proton translocation. In addition, the analogous subunit from the coated vesicle ATPase has been purified and reconstituted into a functional proton channel (161). The 16-kDa polypeptide is an integral membrane protein (69, 120) and is presumably present in multiple copies analogous to the DCCD-binding proteins in other vacuolar-type ATPases and in F_0F_1 ATPases (4, 122). The gene encoding the yeast proteolipid has been cloned and found to code for a 160-amino-acid protein with a calculated molecular mass of 16,352 Da (3, 120). The protein is predicted to have four transmembrane segments. The molecular masses and functions of additional subunits which are likely to form part of the ATPase enzyme have not been reliably determined (15, 69, 70). Although the available nucleotide sequence information for the vacuolar ATPase subunits reveals significant homology to F_0F_1 ATPases, there is a much stronger degree of conservation among vacuolar-type ATPases even from widely divergent organisms (20, 120, 122).

Functions of Vacuole Acidification

Amino acid and ion transport. The vacuolar ATPase utilizes the energy generated by hydrolysis of ATP to pump protons into the vacuole lumen. This results in a calculated electrochemical potential difference of protons on the order of 180 mV contributing to both a decreased pH and a membrane potential of approximately 75 mV for *S. cerevi-*

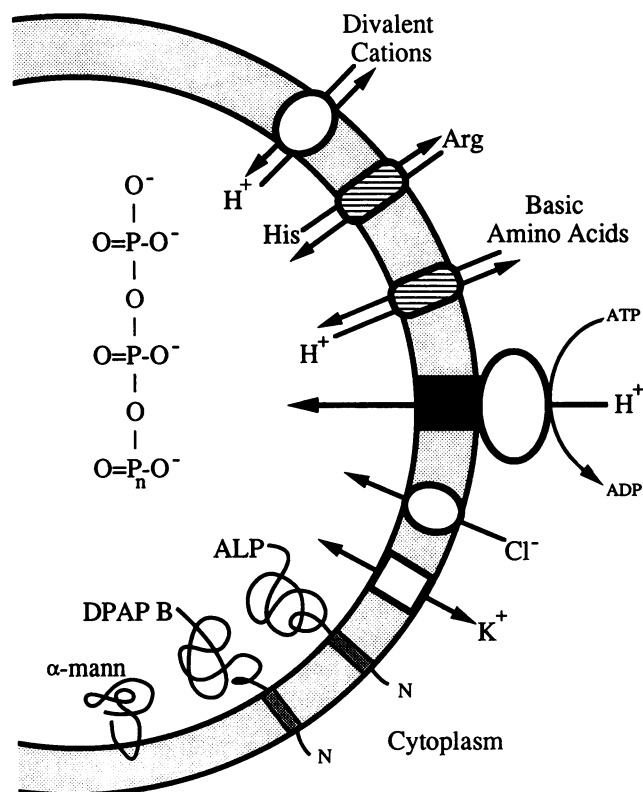


FIG. 5. Overview of the vacuolar membrane and vacuolar transport systems. The H^+ -ATPase functions as the primary proton pump and generates an electrochemical potential that is used to drive other transport systems. The activity of the proton pump may be regulated through specific ion channels in the vacuolar membrane. The role of polyphosphate and details of the various transport systems are described in the text.

siae and 25 to 40 mV for *N. crassa* (14, 68). The electrochemical potential is able to drive amino acid and ion transport (35, 68, 124) (Fig. 5). Since protonophores block transport into the vacuole, ATP hydrolysis itself is not sufficient for the transport process, but must be coupled to the generation of a proton gradient (14). The primary mechanism for transport of storage molecules into the vacuole appears to rely on a proton antiport system (see Compartmentalization of Metabolites). Although the vacuolar ATPase is the major energy donor for these transport systems, there is some evidence that a pyrophosphatase activity is associated with the vacuole membrane in *S. carlsbergensis*, which may be responsible for a PP_i -dependent formation of a pH gradient (97). This would be similar to the function of vacuolar pyrophosphatase of sugar beet taproots (55). Pyrophosphatase activity in *S. cerevisiae* is not associated with the vacuole, but a substantial portion of the endo- and exopolyphosphatase activity is recovered in a vacuole membrane fraction (191).

The pH and electrical potential differences across the vacuolar membrane may be regulated through the interactions of a membrane potential-dependent cation channel (178), chloride transport systems (176), and the vacuolar ATPase (3). The ΔpH may be regulated by modulating the membrane potential; a positive membrane potential inside the vacuole inhibits proton uptake (115). By altering the ion conductivity of the vacuolar membrane, proton uptake ac-

tivity can be blocked or enhanced, while the ATPase activity is relatively unaffected (115).

Precursor maturation. The reduced pH of the vacuole has been implicated in the processing of precursor proteins by triggering the autocatalytic maturation of proPrA (195). This proposal was based on the low pH values predicted for the vacuole and on the observation that proPrA is homologous to pepsinogen, a zymogen that has been shown to undergo pH-induced conformational changes (60). Recent reports on the vacuolar pH, however, suggest that it is not acidified to the same extent as the lysosome; the lysosomal pH may be as low as 4.5 to 5.0, whereas that of the vacuole is closer to 6.0 (90, 107, 118, 137). It is not clear whether the relatively moderate difference between the vacuolar pH and that of a prevacuolar compartment is sufficient to promote a change in protein conformation. Mutants that fail to acidify the vacuole, however, do accumulate precursor forms of vacuolar hydrolases (8, 148). It is necessary to determine whether these precursor proteins are located in the vacuole before assessing the role of pH in precursor processing.

Sorting. The acidification of intracellular compartments plays a role in a variety of intracellular processes (reviewed in reference 107) including protein sorting. One of the best characterized of these is the low-pH-induced dissociation of ligands from their receptors during receptor-mediated endocytosis and lysosomal protein targeting. The observation that some mutants which mis-sort vacuolar proteins are defective in maintaining the proper pH of the vacuole suggested a role for acidification in vacuolar protein sorting. The effect of vacuole acidification on protein sorting has been examined by (i) inhibiting the vacuolar ATPase, the enzyme primarily responsible for generating the proton gradient and electrochemical potential; (ii) dissipating or neutralizing the proton gradient; and (iii) deleting structural genes which encode subunits of the vacuolar ATPase.

Bafilomycin A₁ is a specific and potent inhibitor of the vacuolar ATPase (19, 70, 148). Treatment of yeast cells with bafilomycin A₁ causes an increase in vacuolar pH which can be demonstrated by the abolition of quinacrine accumulation in the vacuole (8). Inhibition of the vacuolar ATPase by bafilomycin A₁ causes precursor accumulation and mis-sorting of CPY, PrA, and PrB (8; D. Klionsky and S. Emr, unpublished observations). Although the reduced vacuolar pH may play a role in promoting the processing of precursor proteins, the precursor accumulation seen in the presence of bafilomycin A₁ is not due simply to the inability to proteolytically process zymogens. First, a substantial fraction of the vacuolar hydrolases are secreted from the cell, similar to the situation with vacuolar protein-sorting mutants. Second, the increase in vacuolar pH caused by bafilomycin A₁ had no effect on the processing of ALP (82), suggesting that the proteolytic maturation capacity of the vacuole was intact. Proper sorting of ALP in the presence of bafilomycin A₁ is similar to the result seen for the sorting of DPAP B in acidification-defective mutants. DPAP B is correctly localized to the vacuole in strains that have extremely reduced levels of vacuolar ATPase activity (69).

Lysosomotropic or acidotropic weak bases can be used to raise the vacuolar pH owing to the neutralization of protons (107). Protonophores can also be used to eliminate the pH gradient by permitting the equilibration of protons across the vacuolar membrane. Treatment of yeast cells with ammonium acetate, ammonium chloride, or carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) results in precursor accumulation and mis-sorting of vacuolar proteins (8, 148). It should be noted that these experiments do not differentiate

between effects of acidification of the vacuole and that of other components of the vacuolar system including the ER, Golgi complex, and endocytic or other prevacuolar compartments. Weak bases will accumulate in and neutralize any acidic compartment. CCCP will also act in a nonspecific manner. Although bafilomycin A₁ is relatively specific for vacuolar ATPase, this type of ATPase may be involved in acidification of the entire vacuolar system. A Golgi ATPase from rat liver was recently purified and shown to be related to the vacuolar-type ATPases (117). This Golgi ATPase is inhibited by bafilomycin A₁ although its activity accounts for only a small fraction of the total ATPase in the Golgi complex. Since the *trans* Golgi is presumed to be the site of vacuolar protein sorting, acidification of this organelle may be important for proper localization of vacuolar proteins.

One model for the role of acidification in vacuolar protein sorting is that the lower pH encountered in the vacuole or prevacuolar compartment allows a receptor to dissociate from vacuolar proteins and recycle back to the Golgi complex, analogous to the receptor recycling involved in mannose 6-phosphate receptor-mediated sorting of lysosomal enzymes. Although the presence of receptors for vacuolar proteins has not been clearly established, one feature of this proposal is that it could explain the differential sorting of soluble and membrane-associated proteins under conditions of elevated vacuolar pH. Since membrane proteins appear to utilize nonluminal sorting components (see Sorting Signals), they presumably do not rely on low pH-induced changes in receptor affinity. Soluble proteins which rely on luminal sorting components, however, would be affected by changes in the luminal pH. If the ligand-receptor complexes are unable to dissociate, the available receptors will become saturated, resulting in secretion of any additional soluble vacuolar proteins by the default pathway. Even though the vacuolar pH may be much higher than that determined for the lysosome, acid-releasable ligands can dissociate at a pH of approximately 6.0 (107), similar to the predicted vacuolar pH (90, 118, 137). A definitive role of the vacuolar pH in protein sorting will be best addressed by using mutants that specifically affect the generation and maintenance of the vacuolar pH. Current work involving the disruption of genes encoding vacuolar ATPase subunits should provide definitive answers specifically regarding the role of the vacuolar ATPase in vacuolar protein sorting (3, 147). Initial studies indicate that a disruption of the genes encoding either the 16-kDa (121) or 57-kDa (121; T. Stevens, personal communication) ATPase subunits prevents vacuolar accumulation of quinacrine and results in the accumulation of precursor CPY, presumably reflecting a vacuolar protein-sorting defect. These results indicate a role for the vacuolar ATPase in vacuole acidification and protein sorting.

Assembly of ATPase

All of the vacuolar ATPases that have been purified have at least three subunits and probably four or five additional polypeptides associated with the final enzyme (15, 16, 69, 70, 96, 116). Radiation inactivation studies suggest that the ATPase complex capable of steady-state ATP hydrolysis has a molecular mass of 410 to 530 kDa (16, 57, 169). Kinetic analyses also indicate catalytic-site cooperativity similar to that of the F₀F₁ ATPases (57, 74, 169). For cooperative interactions to occur, steady-state hydrolysis must involve an integrated multisubunit structure (57). At present, there are few data available on the stoichiometry of all the subunits in the complex or the minimal active domain. The

N. crassa 67- and 57-kDa polypeptides appear to be purified in a 2:1 ratio (16). Analysis of a complex released by KNO_3 (discussed below) led to a proposed stoichiometry of $(67)_3:(57)_3:30:16$ (15). A complex of the *S. cerevisiae* enzyme that is capable of single-site hydrolysis has a molecular mass of approximately 100 kDa (57, 169). Taking into account the apparent molecular masses of the purified subunits, this value suggests that the two major subunits may be sufficient for single-site ATP hydrolysis. This would be similar to the situation with the F_1F_0 ATPases, for which a minimal complex of ATPase subunits $\alpha_1:\beta_1:\gamma_1$ is sufficient for hydrolytic activity (45).

The catalytic site of the enzyme is accessible to a membrane-impermeable substrate (68), suggesting that it is on the cytoplasmic surface of the vacuolar membrane. This orientation is also inferred from the function of the ATPase in acidifying the interior of the vacuole and establishing an electrochemical gradient across the vacuolar membrane with the lumen positive in relation to the cytoplasm. In addition, the large subunits from *S. cerevisiae* and *N. crassa* do not appear to have signal sequences or hydrophobic membrane-spanning domains and are extractable by alkaline Na_2CO_3 (13, 20, 69, 70, 119), suggesting that they are peripheral membrane proteins. Finally, indirect immunofluorescence with monoclonal antibodies to the 69-kDa subunit confirms the cytoplasmic location of the catalytic sector (3). Recent experiments in several laboratories have also presented evidence that the vacuolar ATPase may be assembled in a bipartite structure similar to the F_0F_1 ATPases, having an integral membrane sector forming the proton channel and a peripheral membrane (F_1 -like)-catalytic domain (15, 70, 116). The vacuolar-type ATPase from chromaffin granules undergoes an ATP-dependent cold inactivation, resulting in the release of a 400- to 500-kDa complex composed of five different polypeptides (116). Similarly, an ATP-dependent removal of peripheral membrane subunits by KNO_3 was demonstrated with *N. crassa* (15) and *S. cerevisiae* (69, 70). In *N. crassa*, treatment with KNO_3 results in the coordinate release of four to six subunits which behave as an aggregate of 440 kDa. Additional evidence for an F_1 -like structure is provided by electron microscopy, which reveals the presence of ball-and-stalk structures typical of F_0F_1 ATPases, which were removed with KNO_3 (15). In *S. cerevisiae*, KNO_3 stripping results in complexes of 60 to 240 kDa, suggesting a greater degree of dissociation (70). The presence of large complexes released from the membrane by cold inactivation or KNO_3 indicates that the peripheral membrane subunits may be arranged in an F_1 -like structure. Unlike the F_1 of F_0F_1 ATPases, none of these complexes from the vacuolar ATPases retain hydrolytic activity (15, 70, 116).

An obvious question arises concerning the assembly of the ATPase and, in particular, the cytoplasmic domain. The available nucleotide sequence data fail to reveal the presence of signal sequences or transmembrane domains on the major subunits (13, 20, 69, 119), and the 69- and 57-kDa polypeptides do not undergo N-linked glycosylation (69). These observations suggest that the subunits which make up the cytoplasmic domain of the ATPase may not travel through the secretory pathway. In contrast, the DCCD-binding protein behaves as a proteolipid and is an integral membrane protein (69). This subunit, which is involved in forming the proton channel, and any other unidentified components of the membrane sector are likely to utilize the early stages of the secretory pathway. If the peripheral and integral membrane subunits of the ATPase arrive at the vacuole via

separate mechanisms, assembly becomes a more complex issue. Do the peripheral membrane proteins, including the catalytic segments, assemble into a complex as soon as they are synthesized (69)? If such a complex forms, it may be capable of ATP hydrolysis. This may imply the presence of an inhibitory subunit similar to that found in *Escherichia coli*, for which it has been demonstrated that the F_1 can assemble into a functional complex in the absence of the membrane domain (81, 84). Evidence for some type of regulated assembly is seen with certain mutants that show greatly reduced levels of ATPase activity and a decrease in the association of the 69- and 57-kDa subunits with the vacuolar membrane, even though these subunits are present at wild-type levels (148). The same questions arise concerning the assembly of the proton channel. Can the integral membrane subunits associate with each other to form a functional proton channel in the absence of the peripheral membrane polypeptides? Again, this type of independent assembly has been demonstrated in *E. coli* (5). Since the two functional domains may arrive at the vacuole by separate pathways, are there temporal controls to coordinate the synthesis of the different subunits? Are the levels of synthesis controlled to ensure the appropriate stoichiometric production of the various polypeptides? If the peripheral membrane subunits do not transit through the secretory pathway, how are they targeted to the vacuole? Finally, since there are single copies of the ATPase genes, and the same gene products may be localized in different compartments of the vacuolar system, including the Golgi complex, how are they targeted to the correct locations? It is clear that many questions remain concerning the biogenesis of the ATPase complex. A coordinated genetic, biochemical, and molecular biological approach is being applied to this problem and should begin to provide many of the answers.

Mutations Affecting Vacuole Acidification

Acidification of the vacuolar system clearly plays a role in protein sorting (see Functions of Vacuole Acidification; Sorting). A logical corollary is that some mutants which mis-sort vacuolar proteins may be defective in establishing or maintaining the correct vacuolar pH. A genetic analysis of the mechanism(s) involved in regulating the vacuolar pH should prove useful in further defining the *in vivo* roles of vacuole acidification. Two major approaches have been followed in initiating this type of analysis. The first relies on the screening of existing mis-sorting mutants to identify those which are acidification defective, and the second involves the isolation of new mutants which are isolated on this basis directly.

The relative acidification of the vacuole can be assessed by a variety of methods (reviewed in references 107 and 179). One of the most frequently used techniques involves labeling the vacuole with the weak base quinacrine (179). Quinacrine is able to diffuse across the vacuolar membrane because of its lipophilic nature, but once exposed to the low pH of the vacuolar lumen it becomes protonated and is unable to leave the organelle. In wild-type cells, the vacuole is clearly labeled with quinacrine (179). The selection of mutants that mis-sort vacuolar proteins is described above (see Mutants Defective in Vacuolar Protein Sorting). These mutants were analyzed with regard to quinacrine labeling, and several were identified that were unable to concentrate the dye within the vacuole, indicating a defect in vacuole acidification. The *vpt10*, *vpt13*, and *vpt24* (8), *vpl3* and *vpl6* (148), and *pep12* (137) mutants all show little or no staining

with quinacrine. Although these mutants were isolated independently and through different selection procedures, there is evidence that *vpt13*, *vpl6*, and *pep12* are allelic. Further analyses of these mutants has provided additional insights into the function of vacuole acidification. The *vpt13* mutant displays increased sensitivity to low-pH media relative to a wild-type strain or other *vpt* mutants (8). This may suggest a role of the vacuole in regulating the intracellular pH. The *vpl3* and *vpl6* mutants are deficient in vacuolar ATPase activity and exhibit reduced levels of at least two of the ATPase subunits in isolated vacuole membranes (148). Since these subunits are present at wild-type levels in total-cell extracts from these mutants, the mutations may affect assembly and/or sorting of these proteins. Although the vacuole in *pep12* mutant cells does not accumulate quinacrine, there appears to be only a modest change in the vacuolar pH (approximately 0.1 pH unit) relative to the wild-type vacuole (137). Although the exact nature of these different mutations has not yet been determined, it is clear that they all affect vacuolar protein sorting. With regard to vacuole acidification, then, it is not known whether the primary defect is in acidification which leads to missorting, or in sorting which leads to defective acidification.

Two direct approaches are being used to generate new mutants that affect vacuole acidification. One method relies on a fluorescence ratio assay for measuring vacuolar pH to identify mutants after labeling cells with 6-carboxyfluorescein. By using this technique, a recessive mutant was identified (designated *vph1-1*) that is defective in vacuole acidification and maintains the vacuolar pH at 6.9 (137). To directly analyze the function of the vacuolar ATPase, workers in the laboratories of Stevens, Nelson, and Anraku have begun to clone and disrupt the genes encoding the ATPase subunits. These genes have been designated *VAT* (147) and *VMA* (3). Strains carrying a disruption of the gene encoding either the 16- or 57-kDa subunit of the ATPase are viable, indicating that these genes are nonessential (121, 147). This is in agreement with the observation that the *vph1* mutant has a near-normal growth rate. Proper vacuole acidification is apparently not required for vegetative growth. This is not surprising, since no vacuolar functions essential for vegetative growth have been identified. Interestingly, recent evidence suggests that deletions of ATPase subunits result in conditional lethality that may be influenced by the pH of the medium (121). This may be similar to the temperature-dependent conditional lethality seen with strains having deletions of certain *VPS* genes (see Mutants Defective in Vacuolar Protein Sorting; *vpt* Mutants). These results may indicate a requirement for maintenance of the pH of the vacuolar system under stress conditions.

COMPARTMENTALIZATION OF METABOLITES

One of the most prominent features of eucaryotic cells is the reliance on subcellular compartmentalization. The presence of distinct membrane-enclosed organelles allows the cell to spatially separate otherwise competing reactions. The regulation of various catabolic and anabolic processes may also be mediated simply by compartmentalizing and restricting the appropriate substrates. In addition, fine levels of control may be exerted by modulating the concentrations of physiologically important ions. The role of the vacuole in sequestering many of the major hydrolases of the cell is well appreciated. No less important, however, are its roles as the main storage organelle for a variety of metabolically important compounds and ions. Even a brief examination of the

function of the vacuole in this regard, however, makes it clear that its role as a storage organelle is not a passive one (Fig. 5). The vacuole is involved in the active and precise homeostatic control over the cytosolic access to, and concentration of, many different constituents.

Role of the Vacuolar ATPase in Metabolite Transport

The vacuolar ATPase is the primary enzyme involved in generating an electrochemical potential difference of protons across the vacuolar membrane. The role of the ATPase in providing the energy for transport processes is demonstrated by the sensitivity of the transport reactions to inhibitors of the vacuolar ATPase such as DCCD, KNO_3 , and KSCN (27, 125, 185, 198) and the requirement of ATP for the reaction to proceed (124, 125, 185, 198). In addition, reductions in vacuolar ATPase activity are correlated with decreased vacuolar storage capability (27). The ATPase uses the energy derived from ATP hydrolysis to generate both a proton gradient (ΔpH) and an electrical potential (E_m) across the vacuolar membrane. The electrical potential does not appear to play an obligate role in most transport processes, since they are not inhibited by valinomycin (124, 125). Valinomycin can in fact be stimulatory, most probably because of an alleviation of inhibition resulting from the increased charge separation generated by an electrogenic transport process (125). The proton gradient is the primary driving force for the transport of most metabolites. Protonophore uncouplers such as CCCP and SF6847 and the ionophore nigericin block many transport processes (124, 125, 150, 185, 198). Accordingly, transport of arginine and other amino acids, Ca^{2+} , P_i , Zn^{2+} , Mg^{2+} , and other ions is proposed to occur via H^+ antiporters (124, 125, 129, 150, 198).

Amino Acid Transport and Storage

The presence of two distinguishable amino acid pools, a large pool with a low metabolic turnover and a small pool with a high turnover, has been noted for some time. The large pool was identified as being vacuolar and contains primarily basic amino acids such as arginine (160, 182, 187, 189). The metabolism and compartmentalization of arginine have been thoroughly reviewed by Davis (31), and transport of *S*-adenosyl-L-methionine has been covered by Schwencke and de Robichon-Szulmajster (153). In this review, we will highlight the main points of amino acid transport and storage, including the most recent research in these areas.

Amino acid transport into the vacuole is mediated by a number of transport systems in the vacuolar membrane. Since the transport reactions show saturable kinetics, they presumably reflect interactions with specific protein channels or carriers (126, 151). The differing specificities and kinetic properties have allowed the identification of eight independent transporters in vacuolar vesicles from *S. cerevisiae* (150). H^+ /amino acid antiport systems are present for arginine, arginine-lysine, histidine, phenylalanine-tryptophan, tyrosine, glutamine-asparagine, and isoleucine-leucine. An additional arginine-histidine exchange mechanism that utilizes the chemical potential of the histidine concentration gradient was also detected (150, 151). The presence of these transport systems is in agreement with earlier findings that vacuoles and vacuolar vesicles accumulated primarily these amino acids (124, 172), as well as more recent analyses of vacuolar amino acid pools in Cu^{2+} -permeabilized cells (79). The utilization of three separate systems for sequestering arginine, the amino acid with the

highest nitrogen content, points to the importance of the vacuole as a nitrogen reserve. Research on amino acid transport in *N. crassa* and *S. carlsbergensis* has focused largely on arginine uptake, which also occurs by H^+ /arginine antiport (129, 198). The carrier in *N. crassa* appears to be arginine specific, and there is no evidence for an arginine-lysine transporter as seen in *S. cerevisiae* (198). A protein that is likely to be the arginine carrier has been identified in *N. crassa* by labeling with a reactive arginine derivative (134). The putative carrier protein has a molecular mass of approximately 40 kDa and appears to be membrane associated. Acidic amino acids are not accumulated in the vacuole but, instead, are located almost exclusively in the cytosol, confirming that the presence of particular amino acids in the vacuole is due to specific uptake processes (58, 79, 124, 187). Amino acids are taken up against a concentration gradient, and most are accumulated in the vacuole at levels 5- to 40-fold higher than the corresponding cytosolic concentration (124, 150). Although the size and composition of the cytosolic amino acid pool stay relatively constant, the size and composition of the vacuolar pool vary widely depending on the available nutrients and growth conditions (58, 79). Arginine normally makes up 25 to 30% of the basic amino acid pool in *N. crassa* and *S. cerevisiae* (28, 38). When arginine is the sole nitrogen source, however, it can account for 85% of the basic amino acids in the vacuole (28). Similar effects on the vacuolar, but not the cytosolic, concentrations of ornithine, citrulline, lysine, and histidine are seen when these compounds are added to the growth medium (58, 79).

Although it has been well documented that amino acid uptake is an energy-requiring process (35, 124, 129, 198), the means by which amino acids are retained against a concentration gradient are not as well understood. It has been proposed that polyphosphate serves as a cation trap and forms complexes that are involved in metabolite retention (38). These types of complexes are likely to exist and can be demonstrated to occur in vitro (28, 109). In *S. cerevisiae*, there is generally a stoichiometric correlation between the amounts of arginine and polyphosphates that are accumulated in the vacuole (38). Although polyphosphate may allow larger concentrations of arginine to be accumulated (38, 198), the two pools are, or can be, independently regulated in both *N. crassa* and yeasts (29, 38). Experiments in which the polyphosphate level is reduced by phosphate starvation show that polyphosphates are not required for vacuolar amino acid uptake or retention in either organism (28, 38). The simplest explanation for retention is that the vacuolar membrane is essentially impermeable to cations (28). Once taken up by an active transport process, cations are retained without further expenditure of energy until they are needed.

Since vacuoles serve as stores for numerous metabolites, there must be specific mechanisms for triggering the release of these substances into the cytoplasm under conditions where they become limiting. Basic amino acids, especially arginine, serve as nitrogen reserves. As expected, nitrogen starvation causes mobilization of the vacuolar arginine pool, resulting in increased levels of cytosolic arginine (79, 91, 92). Similarly, limitation of glutamine also results in the release of vacuolar arginine (92). In this case, arginine release is not a general response to amino acid starvation, since it is not elicited by limiting the proline concentration, even though proline is a breakdown product of arginine degradation (31). In fact, arginine is seen to accumulate in the vacuole during proline starvation. Inhibitors of glycolysis also lead to arginine mobilization (35), but, as is the case with glutamine and nitrogen starvation, the actual effector has not been identi-

fied. The observation that respiratory inhibitors or uncouplers block vacuolar release of arginine (35) suggests an energy requirement for efflux.

Inorganic Ion Transport and Storage

It is essential for the cell to regulate the cytosolic ion concentration for several reasons: (i) some ions, such as Si^{2+} , Co^{2+} and Pb^{2+} , are potentially toxic and must be removed from the cytosol; (ii) physiologically useful ions including Ca^{2+} , Mg^{2+} , and Zn^{2+} may become harmful at excess concentrations; and (iii) precise controls of ion concentrations must be maintained if the ions are to be useful in regulatory processes (27, 139, 184). As with amino acids, the vacuole displays selective uptake and storage of particular cations. K^+ and Na^+ , for example, are minor constituents of the vacuolar pools of most organisms even though they are major cytosolic cations (28, 58). The vacuolar uptake of many ions is proposed to occur by H^+ antiport. The activity of the vacuolar ATPase is stimulated by the presence of several ions, and these ions can inhibit amino acid uptake, presumably as the result of transport-induced reductions in the proton gradient (125, 129). There is evidence that Ca^{2+} transport in *S. cerevisiae*, *S. carlsbergensis*, and *N. crassa* is driven by the ATP-dependent formation of a proton gradient (27, 125, 129). The same is true of Zn^{2+} uptake in yeasts (129, 185). A variety of other ions including iron, Mn^{2+} , Co^{2+} , Ni^{2+} , and P_i may also be accumulated in the vacuole, but their uptake properties have not been as well characterized (129, 130, 184). There is substantial evidence that vacuolar cations interact with polyphosphate both in vitro and in vivo (39, 109, 125, 139). In *S. cerevisiae*, the levels of vacuolar inclusions which are presumably due to Ca^{2+} -polyphosphate complexes vary with the level of available polyphosphate (126). Retention of Ca^{2+} is not due solely to trapping by polyphosphate, however, as seen by the release of vacuolar Ca^{2+} upon the addition of proton or Ca^{2+} ionophores (125).

K^+ plays a role in maintaining the ionic and osmotic environment in the cytoplasm, but it may also be involved in the formation of vacuolar ion pools (126). The addition of KCl to the medium results in Ca^{2+} efflux from the vacuole and an increase in vacuolar K^+ (39). A similar coupling of arginine efflux with K^+ influx is also observed (79). There is some evidence that in *S. carlsbergensis*, K^+ is accumulated in the vacuole against a concentration gradient (130). A vacuolar K^+ concentration gradient can also be attained in Cu^{2+} -treated yeast cells and may be able to supply the driving force for transport processes (126). These observations are interesting considering the identification of a membrane potential-dependent cation channel capable of conducting K^+ and other monovalent cations (178).

Polyphosphates

Polyphosphates are the only macromolecular anion in the vacuole (28), and their roles in basic amino acid and cation retention (see above) and osmoregulation (see below) are discussed elsewhere in this review. Polyphosphate serves in a storage capacity for P_i and is located only in the vacuole (29, 38, 59, 170). The polyphosphate chains range in size from 3 to 260 units, with most being 3 to 45 or 7 to 20 units in *N. crassa* and *S. cerevisiae*, respectively (47, 170, 172). These sizes must be treated with some caution, however, because of the potential action of phosphatases during purification. The chain length may be an important factor in

the stability of polyphosphate-cation complexes (109) and may also be related to the growth stage (47). The addition of ammonium salts to the medium causes polyphosphate hydrolysis and a subsequent increase in cytosolic phosphate (47). This may reflect a nitrogen-induced efflux of metabolically useful phosphate reserves.

pH and Osmoregulation

The vacuole is an acidic organelle, and maintenance of the vacuolar pH is important for a number of cellular functions (see Functions of Vacuole Acidification). The vacuolar pH in *S. cerevisiae* and *C. albicans* has been shown to vary with the growth stage (22, 47). The vacuolar and intracellular pH values undergo relatively small changes in response to substantial alterations in the extracellular pH when cells are in the stationary phase of growth (47, 118). The observation that some yeast mutants which lack a normal vacuole are pH sensitive suggests that vacuoles may play a role in homeostasis of the intracellular pH (8). These mutants also show some degree of osmosensitivity, indicating an additional role in osmoregulation. In addition, other mutants defective in vacuolar protein sorting have extremely large vacuoles (8), which may reflect a defect in osmoregulatory capabilities. Polyphosphate formation plays some role in osmoregulation by reducing the osmotic pressure of P_i , and interaction with polyphosphate reduces the osmotic activity of vacuolar amino acids (28, 38). Certainly some of the ions and amino acids in the vacuole are in an osmotically active form (124, 125); however, vacuole size does not change during nitrogen starvation in *S. cerevisiae*, even though there is a rapid decrease in the vacuolar arginine pool (79). This may be due to the approximately stoichiometric increase in vacuolar K^+ which accompanies the arginine efflux. Although regulation of osmotic or electrical potential differences is not well understood, some control may be afforded by the membrane potential-dependent cation channel (178). Since the vacuole and cytosol are isotonic (28, 37), this channel may be an osmotic regulator which acts to balance the osmotic potential difference resulting from the uptake of cations into the vacuole (178). Growth of *N. crassa* on arginine as the sole carbon source or under conditions of phosphate starvation results in large vacuoles (28). In this case, the excess cationic charge results in increased osmotic pressure due to small ions, which are needed for charge neutralization.

Regulation of Transport

Regulation of the transport systems is not well understood, but kinetic analyses provide some insight into the control of metabolite uptake. In *S. cerevisiae*, *S. carlsbergensis*, and *N. crassa*, the cation antiporters have K_m values similar to the concentrations of those cations in the cytosol (124, 129, 150, 198). This suggests that transport occurs until the cytosolic level reaches the K_m value. In addition, the *S. carlsbergensis* plasma membrane ATPase is inhibited by Mg^{2+} concentrations that activate the vacuolar ATPase and are within the K_m range for the vacuolar Mg^{2+}/H^+ antiporter (129). Similarly, the various H^+ /amino acid antiporters in *S. cerevisiae* have K_m values 10 to 100 times higher than the corresponding values for uptake systems in the plasma membrane (124, 150). This reflects the need to remove these metabolites from the cytosol when their concentrations become too high. One important aspect of the transport systems that is not well understood is the way in which efflux from the vacuole is controlled. Release of arginine, for

example, is triggered by nitrogen or glutamine limitation, but the means by which they exert their effect is not known (91, 92). As with other vacuolar functions, it is necessary to examine the effects of specific mutations to fully understand the physiological roles of metabolite compartmentalization. Recent advances along these lines should provide useful information.

Vacuolar Storage Mutants

Although amino acids and certain ions are critical for various cellular processes, excess levels of these substances can be toxic to the cell. For this reason, homeostatic control of the cytoplasmic concentration of amino acids and ions, carried out by the vacuole, is extremely important. Accordingly, it was reasonable to predict that any mutations which affected the ability of the vacuole to store a particular substance might lead to impaired growth in the presence of a high concentration of that substance. These mutations could exert their effect through a number of possible ways, including (i) inability to transport into or out of the vacuole, (ii) loss of storage capacity, and (iii) impaired regulatory control and loss of homeostasis. In fact, it is likely that there would be considerable overlap among these effects. A defect in the vacuolar ATPase, for example, could prevent the accumulation of amino acids and ions, a transport defect, as well as resulting in the loss of homeostatic control of these metabolites. This type of pleiotropic mutation is demonstrated by the Ca^{2+} -sensitive mutants of *N. crassa* isolated by Cornelius and Nakashima (27). Although these mutants were selected on the basis of their growth sensitivity to high levels of Ca^{2+} , they are not defective in Ca^{2+} -specific transport or storage. Uptake of Ca^{2+} into the vacuoles of these mutants occurred more slowly, consistent with the finding that lower Ca^{2+} concentrations were required for optimal growth. The vacuoles also had reduced levels of arginine and showed a substantial decrease in ATPase activity (27). Because of their pleiotropic nature, it is unlikely that the mutations affect the Ca^{2+}/H^+ antiporter. One possibility would be that they are mutations in the vacuolar ATPase, since an impaired ability to generate a proton gradient would affect the transport of both Ca^{2+} and arginine. Calcium-sensitive mutants of *S. cerevisiae* have also been isolated (127). Analyses of Ca^{2+} content and uptake activity indicate that some of these mutants may be defective in sequestering the intracellular Ca^{2+} pool, suggesting an impaired vacuole. These mutants, designated *cls* type III, also show increased sensitivity to trifluoperazine (TFP). Recent findings indicate that an allele of the yeast gene encoding the 69-kDa subunit of the vacuolar ATPase confers TFP resistance (155; T. Stevens and N. Neff, personal communication). This suggests that mutations leading to TFP sensitivity could simultaneously cause a defect in the function of the vacuolar ATPase. The resulting decrease in the electrochemical potential across the vacuolar membrane, and the corresponding reduction in vacuolar Ca^{2+} uptake activity, may be one explanation for the Ca^{2+} -sensitive phenotype of *cls* mutants. In support of this, a mutation in the TFP resistance gene conferred a calcium-sensitive growth phenotype, although in this case the mutation caused TFP resistance (155).

A similar rationale was used by Kitamoto et al. to identify mutants that were defective in the storage of basic amino acids (78). The catabolic enzymes involved in arginine metabolism are located in the cytosol and have been well characterized (reviewed in reference 31). This cytosolic degradation is an additional way in which arginine is pre-

vented from reaching toxic levels. In contrast, no mechanisms appear to exist for degrading histidine or lysine. This may be the reason why yeast cells grown in the presence of lysine or histidine increase the levels of these amino acids in the vacuole 27- and 42-fold, respectively, compared with a 7-fold increase for arginine (79). If the ability to concentrate these amino acids in the vacuole were impaired as a result of a mutation, the mutants would presumably grow poorly in the presence of high concentrations of lysine or histidine. Mutants displaying a lysine-sensitive growth phenotype were isolated and found to have small vacuolar pools of lysine, histidine, and arginine (175). These mutants are designated *slp1* (for small lysine pool) and had vacuolar levels of basic amino acids that were reduced 30 to 90% compared with the wild type. Since the mutants were defective in the storage of more than one amino acid and the phenotypes result from a single mutation, they are not likely to be specific transport mutants. In addition, the mutants show increased sensitivity to Ca^{2+} and heavy-metal ions, suggesting a more general defect in homeostatic control. A morphological examination reveals the absence of a typical large vacuole in these mutants, accompanied by an increase in vesicular structures. Since the available lysine pool was utilizable, it was suggested that the vesicles could be vacuole related and still retain some normal vacuolar functions (78). Similarly, analysis of another vacuole-deficient mutant, the *end1* mutant (25), revealed an inability to accumulate arginine and polyphosphate (183). This lack of nitrogen and phosphate reserves results in decreased growth rates during starvation conditions compared with a wild-type strain. The gene complementing the *slp1* mutation was recently cloned and sequenced (175). The deduced amino acid sequence predicts a protein of 691 residues with an estimated molecular mass of approximately 79 kDa, and it does not appear to have any transmembrane domains. *SLP1* is a nonessential gene, but loss of *SLP1* function causes a two- to threefold decrease in the growth rate. The morphological defect exhibited by the *slp1* mutant is similar to the defect seen in certain *vps* mutants (8). The class C mutants isolated by Robinson et al. (143) also lack a single large vacuole and contain numerous vesicular structures (see Mutants Defective in Vacuolar Protein Sorting; *vpt* Mutants). Comparison of the nucleotide sequence of *SLP1* with that of cloned *VPS* genes reveals that *SLP1* and *VPS33* are identical (175; L. Banta and S. Emr, unpublished results). This explains the observation that *slp1* mutants accumulate precursor forms of vacuolar proteases (78). Interestingly, *SLP1* had previously been identified as being allelic to *VAM5*, a vacuole morphology mutant (77). In addition, *vam5* is allelic to *cls14*, a calcium-sensitive mutant (127, 177). Similarly, the vacuole-deficient *end1* mutant described above is allelic to *vps11* (143), *vam1* (177), and *cls13* (127, 177). The same genes have been identified by screening for defects in protein sorting, amino acid and Ca^{2+} storage and homeostasis, and vacuole morphology. This allows us to pose a question which is a general one when dealing with vacuolar mutants and arises because of the many overlapping functions carried out by the vacuole. What is the nature of the primary defect; and does this mutation affect vacuole morphology, resulting in decreased storage capability and vacuolar protein missorting owing to the absence of a proper target, or is it a sorting defect which prevents normal vacuole formation because of the missorting of a protein critical in morphogenesis? An answer to this question may be provided by a detailed analysis of strains with mutations that affect vacuole morphology. Certain alleles of the *vps33* gene, for example, lead

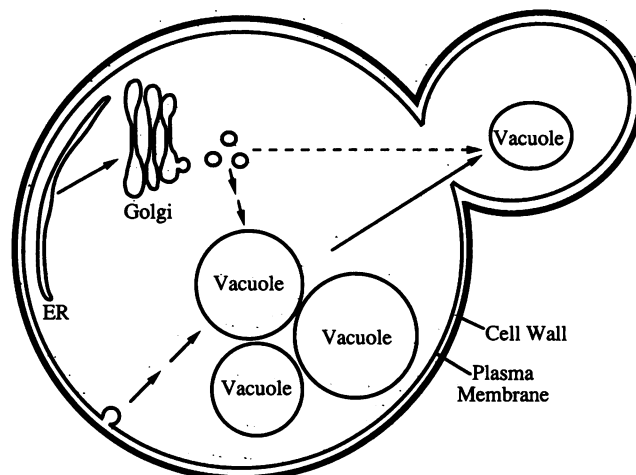


FIG. 6. Biogenesis of the fungal vacuole. The daughter cell or bud inherits a substantial portion of its vacuolar contents from the mother cell. The role of the Golgi complex and endocytosis in the development and maintenance of the vacuolar structure is discussed in the text.

to the absence of a normal vacuole (class C; see Mutants Defective in Vacuolar Protein Sorting), whereas other *vps33* alleles contain morphologically normal vacuoles. Vacuolar hydrolases, however, are still missorted by each of the *vps33* mutants (Banta and Emr, unpublished). This observation suggests that, at least in this case, the primary defect is due to missorting.

VACUOLE BIOGENESIS

Studies of the vacuolar compartment in the budding yeast *S. cerevisiae* have provided some insight into the mechanisms regulating the assembly and inheritance of this organelle (Fig. 6). The wild-type vacuolar compartment is a very dynamic structure, which is capable of undergoing rapid changes in its morphological appearance. Studies of the biogenesis of this organelle have been complicated by observations which suggest that vacuolar structure is influenced by the preparative techniques used during the analysis (138). The yeast cell vacuole is easily detected at a very early stage in the growth cycle. When the daughter bud is only a fraction of the size of the mother cell, a vacuole is already present within the bud (179). Experiments with stable fluorophore labeling of yeast vacuoles suggest that the daughter cell inherits a substantial portion of its vacuolar contents from the mother cell (179, 181). Such vacuolar inheritance had been suggested by the pronounced phenotypic lag observed in the expression of the *Pep4* mutant phenotype in newly sporulated cells (199). However, the cellular mechanisms responsible for faithful vacuole partitioning during cell division are poorly understood. Early experiments with synchronized yeast cultures had indicated that the vacuole undergoes a cyclic pattern of fragmentation and coalescence during the yeast cell cycle (154, 188). Upon bud emergence, a rapid transition from large to multiple, small vacuoles was observed. It was suggested that the small vacuolar structures were then distributed between the mother and daughter cells. However, a more recent study with asynchronous cultures has suggested a very different mechanism of partitioning (179). In this study, the morphology of the vacuolar compartment was observed to be relatively constant

throughout the cell cycle, and most mother and daughter cells possessed a single vacuole. It was proposed that in the absence of vacuole fragmentation, traffic between the mother and daughter cell vacuoles, mediated by either vesicles or tubular connections, is responsible for the observed partitioning of the vacuolar contents (179). The isolation of a *vac1* yeast mutant, which may be defective in this vacuolar partitioning process, has recently been reported (180). It seems unlikely that the cyclic vacuole fragmentation pattern observed in the synchronous-culture experiments was an artifact of the synchronization procedure used, as each study used a different technique to achieve synchrony (154, 179, 188). It is interesting that a fragmented vacuole morphology is observed in several of the *vps* mutants (8, 144) and in yeast cells which have been treated with microtubule-disrupting agents (49). However, it is not known whether the fragmented vacuoles observed under these conditions represent true physiological intermediates in vacuole biogenesis. Although the precise mechanisms of vacuolar segregation are not yet understood, these studies clearly demonstrate that the mother cell vacuole contributes significantly to the vacuolar contents of the newly forming bud.

The vacuolar compartment continues to grow in volume following its initial appearance in the newly emerging bud. The intracellular mechanisms responsible for this observed growth are not well understood. An analysis of yeast mutants defective in the localization of vacuolar proteins has identified mutants which also appear to be defective in vacuole assembly (see Mutants Defective in Vacuolar Protein Sorting). These observations suggest that the biosynthetic pathway delivering vacuolar proteins from the Golgi complex may be contributing to the growth and/or maintenance of the vacuolar structure. Endocytosis may also participate in this process, as is seen in mammalian cells in which endocytic traffic is routed to the lysosomal compartment via specific endosomal intermediates (107). Endocytosis in yeast cells has been examined through an analysis of the internalization of enveloped viruses (99), α -amylase (100), lucifer yellow CH (25, 141), and α -factor (25, 61) by whole cells or spheroplasts. Fluorescein isothiocyanate-dextran has also been used as an endocytic marker, but conflicting reports have appeared regarding the ability of yeast cells to endocytose this macromolecule (32, 99, 136). Genetic and biochemical evidence from these studies indicates that this endocytic traffic contributes to vacuolar content. The dye lucifer yellow CH accumulates within the vacuolar compartment as a result of fluid-phase endocytosis (25, 141). The mating pheromone, α -factor, is bound by a specific cell surface receptor and is subsequently internalized and degraded (61, 141). This degradation of α -factor has been shown to be *PEP4* dependent, suggesting that this breakdown occurs within the lumen of the vacuole (36). Therefore, the growth of the vacuole may involve the coordinate regulation of at least these two different pathways of de novo biosynthetic traffic and endocytosis. An assessment of the contributions of either pathway would be greatly facilitated by the identification of mutants defective in only one component. Thus far, however, no yeast mutants specifically defective in endocytosis have been identified. The *end1* mutant (*end1* is allelic to *vps11*; see Mutants Defective in Vacuolar Protein Sorting), originally identified as being defective in the receptor-mediated endocytosis of α -factor (141), has subsequently been shown to be competent for the uptake of the α -factor pheromone but defective in its degradation (36). Finally, an analysis of clathrin-deficient yeast

cells has indicated that the clathrin heavy chain is not essential either for α -factor uptake or for vacuolar protein delivery (135). Continued analysis of vacuolar protein sorting-defective mutants might provide some insight into the mechanisms regulating vacuole biogenesis (8, 144).

Several different genetic approaches have been successful in identifying mutants which may be defective in specific aspects of vacuole biogenesis (see Mutants Defective in Vacuolar Protein Sorting). A preliminary report has described the isolation of yeast mutants defective in vacuole assembly (77, 177). These vacuole morphology (*vam*) mutants were isolated by visually screening for cells possessing abnormal vacuolar structures (177). Many of the *vam* mutations are allelic to previously isolated vacuolar storage and protein-sorting mutations (175, 177; S. Emr, unpublished observations) (Table 2). Many mutants originally identified as being defective for specific vacuolar functions, such as Ca^{2+} (127) or lysine (78) storage and vacuolar protein sorting (8, 144), have subsequently been shown to possess an abnormal vacuolar compartment. These mutants may therefore define gene functions required for the assembly and/or maintenance of the wild-type vacuolar structure.

CONCLUSION

Perhaps the single most important point that we have tried to convey in this review is that the fungal vacuole is an extremely complex organelle that is involved in a wide variety of functions. The vacuole not only carries out degradative processes, the role most often ascribed to it, but also is the primary storage site for important metabolites such as basic amino acids and polyphosphate, plays a role in osmoregulation, and is involved in the precise homeostatic regulation of cytosolic ion concentration and pH. These many functions necessitate an intricate interaction between the vacuole and the rest of the cell; the vacuole is part of both the secretory and endocytic pathways and is also directly accessible from the cytosol. Although models have been proposed to describe general features of the vacuole and its protein constituents, it is probably not generally useful to think of the vacuole in terms of unifying themes or prototypical proteins. This point is illustrated by examining the diverse ways in which proteins arrive at the vacuole. The secretory pathway is the major route used for the delivery of most hydrolases. This mechanism of delivery is best illustrated by CPY. Even when dealing with delivery through the secretory pathway, however, distinctions must be made between proteins that are soluble and those that transit as membrane-bound forms, such as ALP and DPAP B. Although both of these classes of proteins most probably travel to the vacuole via vesicular carriers, this is probably not true for all of the vacuolar constituents. An interesting alternative may be seen with α -mannosidase, a luminal protein that may be translocated directly across the vacuolar membrane. Other proteins which may not use the secretory pathway include the peripheral membrane subunits of the vacuolar ATPase. Since the integral membrane components of this enzyme are likely to utilize the secretory pathway, an additional complexity is added, as there must be some coordination between delivery and/or assembly of proteins that arrive at the vacuole through separate mechanisms. Similarly, the processing pathways are not as simple as first believed. CPY again provides the classic model of signal peptide removal at the ER followed by cleavage of an N-terminal propeptide upon, or just prior to, arrival in the vacuole. The classic model, however, may not be widely

applicable, even among proteins that undergo PrA-dependent maturation. API, for example, has an N-terminal propeptide but apparently lacks a hydrophobic signal sequence. ALP has an internal uncleaved signal sequence that causes it to remain membrane bound, and its propeptide is removed from the C terminus. PrB has an even more complicated processing scheme involving the cleavage of a large N-terminal segment in the ER followed by two successive proteolytic events later in the delivery process.

Other vacuolar functions besides those involving the hydrolases also show considerable variation. Although the vacuole functions as a storage compartment, this term is too simplistic. An examination of different metabolites reveals tremendous diversity in compartmentalization. The major cellular pool of arginine is kept metabolically inactive, since it is sequestered within the vacuole and is inaccessible to the cytosolic biosynthetic and catabolic enzymes. The reverse situation occurs for trehalose, with the substrate and the degradative enzyme being located in the cytosol and in the vacuole, respectively. Still another example is demonstrated by the storage of polyphosphate, which is localized to the vacuole along with at least some of the enzymes responsible for its degradation. The means by which entry or release of substrates and regulation of degradation are achieved are not well understood. Because of the various roles and properties of the vacuole, it has been possible to isolate mutants which are defective in various vacuolar functions including the storage and uptake of metabolites, regulation of pH, sorting and processing of vacuolar proteins, and vacuole biogenesis. Interestingly, these mutants show a remarkable degree of overlap, suggesting that these functions are not individual, discrete properties of the vacuole but, rather, are closely interrelated.

Many questions remain to be answered about the vacuole and its constituent proteins. The precise characterization of sorting signals used to target proteins to the vacuole has not been achieved. Identification of components of the sorting apparatus, including potential receptors, will rely on continued analyses of missorting mutants. At present, almost all of the data available on vacuolar metabolite transport systems concern their kinetic properties. The purification of vacuolar permeases, analyses of their biosynthesis, and elucidation of control mechanisms await future efforts. Although rapid progress has been made in understanding the vacuolar ATPase, basic questions still remain; these address the subunit composition and stoichiometry as well as more intriguing problems concerning assembly. The role of the vacuole in endocytosis remains largely undefined. Similarly, little is known about vacuolar biogenesis and inheritance. Continued work on the vacuole will further reveal the complex nature of this organelle and the ways in which it is integrally involved in a variety of cellular processes. Clearly, the vacuole will remain a rich and exciting area of research for many years to come. We can also expect that the insights gained from these studies of the fungal vacuole will influence our view of related processes in mammalian and plant cells, which are far less tractable to genetic and molecular dissection.

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